REMARKS

Claims 29-42 and 44-54 are pending in the subject application. By this Amendment, applicant has amended claims 29, 36 and 42.

Support for the amendments to claims 34, 36 and 42 may be found in the specification, *inter alia*, on page 11, lines 6-8 of the subject application.

Accordingly, claims 29-42 and 44-54 are currently pending, of which claims 34, 35, 40, 41, 47, 48, 53 and 54 have been withdrawn pending allowance of the examined claims.

Request for Examination of Claims 34-35, 40-41, 47-48 and 53-54

In the February 25, 2008 Office Action, the Examiner maintained the propriety of the withdrawal of claims 34, 35, 40, 41, 47, 48, 53 and 54 from consideration as being directed to *non-elected species*. The Examiner asserted that in the subject application, since the pending generic claims have not been found allowable applicants are not entitled to examination of additional species other than the specific species elected for examination, but would be so entitled if the generic claims are found allowable, according to 37 C.F.R. 1.141(a).

Applicant's Reply

In response, applicant maintains that for the currently withdrawn claims drawn to species outside of the specific species elected for examination applicant is entitled to have these claims rejoined and allowed once the generic claim is found allowable. In light of the claim amendments set forth above and the remarks which follow applicant maintains the generic claims are allowable and requests the withdrawn claims directed to nonelected species be rejoined and allowed.

requests the examination of additional species pending allowance of the examined claims.

Rejection under 35 U.S.C §112, Second Paragraph

In the February 25, 2008 Office Action, the Examiner rejected claims 29-33, 36-39, 42, 44-46 and 49-52 under 35 U.S.C. 112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 29, 36 and 42

The Examiner asserts that it is unclear as to what the relationship is between the "treatment" and the increase in plasma HDL cholesterol levels is intended by the phrase "accompanied by". The examiner further stated that the claims fail to clearly set forth whether the claimed treatment *per se* affects an increase in plasma HDL cholesterol levels or whether the human subject is experiencing a concomitant increase in plasma HDL cholesterol levels simultaneously with administration of the instantly claimed treatment. The Examiner asserted that one of ordinary skill in the art at the time of the invention would not have been reasonably apprised of the subject matter for which the applicant is presently seeking protection.

Applicant's Reply

In response, applicant respectfully traverses the Examiner's rejection. However, in order to expedite prosecution, and without conceding to the correctness of the Examiner's position, applicant has amended claims 29, 36 and 42 to indicate that "administration of said xenobiotic fatty acid compound (i.e. 3,3,14,14-tetramethyl-hexadecane-1,16-dioic acid) increases plasma levels of HDL cholesterol, so as to thereby treat the syndrome in the human subject for the condition recited in the claim. Applicant also emphasizes that, as summarized by Ford and

Giles in Diabetes Care, Vol 26, Number 3, March 2003 (copy attached hereto as **Exhibit A**), low HDL cholesterol is one of the recognized Metabolic Syndrome criteria, and therefore, administration of a medicament leading to an increase in HDL plasma levels is desired in treating this disorder.

Accordingly, applicant maintains that the pending claims, as amended, are definite and request that this ground of rejection be reconsidered and withdrawn.

Claims 29-33

The Examiner also rejected claims 29-33 under 35 U.S.C. 112, second paragraph. The Examiner alleged that the subject matter intended by the phrase "thrombogenic/fibrinolytic defects" is not clearly set forth in the claims or the specification such that one of ordinary skill in the art at the time of the invention would have been reasonably apprised of the scope of subject matter for which the applicant is seeking protection.

Applicant's Reply

In response, applicant respectfully traverses the Examiner's rejection. However, in order to expedite prosecution, and without conceding to the correctness of the Examiner's position, applicant has amended independent claim 29 so as to no longer recite "thrombogenic/fibrinolytic defects." As a consequence, dependent claims 30-33 also no longer recite "thrombogenic/fibrinolytic defects"

Accordingly, applicant maintains that the pending claims, as amended, are definite and request that this ground of rejection be reconsidered and withdrawn.

Rejection under 35 USC § 103(a)

In the February 25, 2008 Office Action, the Examiner rejected claims 29-33, 36-39, 42, 44-46 and 49-52 under 35 U.S.C. 103(a) as unpatentable over ***Russell et al.*** ("Hypolipidemic Effect of β,β'-Tetramethyl Hexadecanedioic Acid (MEDICA 16) in Hyperlipidemic JCR:LA-Corpulent Rats", *Arteriosclerosis and Thrombosis*, 1991; 11:602-609; already of record), citing to ***Bar-Tana*** ("Long Chain Dicarboxylic Acids: Hypolipidemic, Antiobesity and Antidiabetic Activity", New Antidiabetic Drugs, 1990; already of record) to show a fact, in view of ***Hertz et al.*** ("Mode of Action of Peroxisome Proliferators as Hypolipidemic Drugs", *Journal of Biological Chemistry*, 1995; already of record) and ***Ferrannini et al.*** ("Hypersinsulinemia: The Key Features of a Cardiovascular and Metabolic Syndrome", *Diabetologia*, 1991; already of record).

Applicant's Reply

In response, applicant respectfully traverses the Examiner's rejection. The Examiner asserted that Russell et al. teaches the administration of MEDICA 16 to male and female obese JCR:LA-corpulent rats, wherein MEDICA 16 administration for 14 days demonstrated a reduction in the serum triglyceride concentration and hepatic triglyceride secretion rate following treatment. The Examiner further asserted that Russell et al. supports this reduction in whole serum triglycerides by approximately 80%, as well as a modest decrease in cholesterol following treatment and demonstrates a clear increase in high density lipoprotein (HDL) lipids for female *cp/cp* rats treated with MEDICA 16.

Upon analysis of Table 5, applicant notes that one of ordinary skill in the art would immediately identify the inconsistency of the results reflecting the effect of MEDICA 16 on the total cholesterol. While Table 3 shows a decrease in the total cholesterol, Table 5 presents the opposite effect, i.e. an increase (from 68.0±11 to 80.0±4.1) in the total cholesterol in

response to MEDICA 16 treatment. Moreover, it should be noted that the total sum of the different fractions in response to MEDICA 16 treatment, is higher than the total cholesterol (e.g., 5.6+2.4+81=89, vs. the total indicated as 80).

The effect of MEDICA 16 treatment as indicated by Table 5, is very specific to the female rat of this particular animal model and is not shared by any other rodent or even rats, as will be indicated in detail hereinafter, an effect probably not shared even by male rats in the same model (JCR:LA-corpulent rats).

Still further, analysis of the lipid profile of control animals in this rat model as demonstrated by Table 5, shows a similar profile to that shown by other rodents. Most of the serum total cholesterol (68.0±11.0) is the HDL fraction (40.5±4.3), part of it is the VLDL (13.8±4.2), and only a negligible amount is in the LDL fraction (1.2±0.2). Therefore, it would be expected that any elevation in the total cholesterol level (as shown by Table 5, but not by Table 3), will be reflected in elevation of the HDL fraction that is the main lipid fraction in the rat, which is indeed demonstrated only by Table 5.

Still further, as indicated above, the surprising elevation of HDL in female JCR:LA-corpulent rats shown by Table 5 of the Russell et al. publication cited by the Examiner, is a particular case and is not shared by any other rodents or even rats. As described by Russell et al., a copy of which is attached hereto as **Exhibit B**, a further study performed by the same author using the very same animal model, indicated a significant decrease in the total cholesterol level from 142±46 to 96.5±20.6, of MEDICA 16 treated male rats, as demonstrated Table 2 (page 920). Although the particular fractions are not indicated in this study, since the HDL fraction is the main lipid fraction in rodents, the skilled artisan would necessarily predict that such

reduction will be reflected also in a corresponding reduction of the HDL levels. Bar-Tana et al., 1988, a copy of which is attached hereto as **Exhibit C** discloses the effect of MEDICA 16 on normal rats. As shown by Table 1 (page 434), treatment with MEDICA 16, resulted in a marked reduction of the triglycerides, reduction of the total cholesterol (from 58.0±13.5 to 32.3±6.6), that is mostly reflected by the VLDL fraction (reduction of 23.2±5.3 to 6.1±3.1), and is accompanied by a marked reduction in the HDL levels (from 30.4±4.9 t 20.6±4.2) and elevation of the LDL levels (from 1.6±0.2 to 5.1±2.0). In yet another example, shown by Mayorek et al., 1997, a copy of which is attached hereto as **Exhibit D,** showed that MEDICA 16 leads to decrease in total cholesterol in obese Zucker rats, from 153±10 to 92±11, as indicated by Table 1 (page 1960). As indicated above, since most of the serum cholesterol in rats is in the HDL fraction, it would be recognized by the skilled artisan that these results reflect reduction in HDL in response to MEDICA 16 treatment. Thus, in contrast to the results presented by Table 5 of Russell et al. cited by the Examiner, in normal rats as well as in other rat models, MEDICA 16 leads to a decrease in HDL.

Another example of rodents may be presented by the sand rat model, as disclosed by Ruth Tzur et al., 1988, a copy of which is attached hereto as **Exhibit E**. As shown by Table 1 (page 1619), treatment with MEDICA 16 led to a significant decrease in the plasma cholesterol, from 69.0±7.7 to 39.6±7.0. Since in rodents most of the serum cholesterol is the HDL fraction, the skilled artisan would note that the MEDICA 16 leads to decrease in the HDL cholesterol. In still another rodent model, i.e. the male hamster Mayorek et al., 1993, a copy of which is attached hereto as **Exhibit F,** showed in Table 1 (page 913), that treatment with MEDICA 16 led to reduction in plasma cholesterol (from 266±29 to 141±21), and reflected by a significant reduction in the HDL

levels (from198±19 to 104±10). Thus, two other rodent models showed that MEDICA 16 leads to a significant decrease in HDL.

Taken together, it seems that the elevation in the HDL fraction presented in Table 5 of Russell et al. cited by the Examiner, if not a mistake (in view of the contradicting results of Table 3 of the same publication), is a most a very particular case of female JCR:LA-corpulent rats, that cannot be considered by the skilled artisan as being in any way predictive of what will happen even in other rats, let alone in other rodents, and certainly not in humans.

Even in case the skilled artisan would not be aware of the fact that the lipid profile of rodents and particularly, of this specific rat model is completely different and therefore cannot be considered as appropriate model for lipid disorders in human subjects, the fact that MEDICA 16 treatment results in increase in the LDL levels, indicates that such treatment likely leads to an increase in LDL in human subjects, and therefore teaches away from the possibility of using MEDICA 16 for treating Metabolic Syndrome and dyslipoproteinemia in human subject.

Even in case the skilled artisan would not be aware of the fact that the lipid profile of rodents and particularly, of this specific rat model is completely different and therefore cannot be considered as appropriate model for lipid disorders in human subjects, the fact that MEDICA 16 treatment results in increase in the LDL levels, indicates that such treatment likely leads to an increase in LDL in human subjects, and therefore teaches away from the possibility of using MEDICA 16 for treating Metabolic Syndrome and dyslipoproteinemia in human subject.

As for the Examiner's conclusion that Hertz et al., who teaches that "Aryloxyalkanoic fibrates (e.g. clofibrate (1) and

bezafibrate (2)), substituted long chain dicarboxylic acid (e.g. MEDICA 16 (3,4)), and other amphipathic carboxylates lower plasma triglycerides and cholesterol levels, and some are extensively used in humans as drugs of choice for treating hypertriglyceridemia or combined hypertriglyceridemia/hypercholesterolemia." (para .2, 1.1-6, col. 1, p. 13470), supports the reasonable expectation of success that MEDICA 16 would have had efficacy in treating combined hypertriglyceridemia and hypercholesterolemia, which both were known to characterize the condition of "dyslipoproteinemia" as described by Applicant (see p.10, 1.23-24 of the instant specification). It should be noted that since claims 36-39 are restricted to a method of treating dyslipoproteinemia using a substance leading to increase in HDL, that is not mentioned or even hinted by Hertz et al, combination of this document with Russell et al., which has been shown above as teaching away from the present invention, would not be considered by the skilled artisan as demonstrating the obviousness of applicant's claimed invention.

As for the Examiner's statement that the efficacy of the compound MEDICA 16 in reducing plasma triglycerides and plasma cholesterol and increasing HDL cholesterol would have been reasonably suggestive of the same or a substantially similar level of efficacy in treating Syndrome X, in view of combining Ferrannini et al., with Russell et al. and Hertz et al. Applicant asserts that such a conclusion is not appropriate. More specifically, the Examiner, states that Ferrannini et al., indicates that Syndrome X is characterized by the concomitant occurrence of <u>any one or more of</u> insulin resistance, glucose intolerance, hypertension and dyslipidaemia and therefore concludes that in light of such a teaching, it would have been *prima facie* obvious to one of skilled artisan that the efficacy shown by the compound MEDICA 16 in treating derangement of plasma lipids (i.e. triglycerides and

cholesterol) and increasing "good" HDL cholesterol would necessarily have had efficacy in treating Syndrome X, since Syndrome X was know in the art to be characterized by lipid dysfunction and Russell et al. teaches the efficacy of MEDICA 16 in reducing serum triglycerides and cholesterol and increasing "good" HDL cholesterol.

In response, it should be first noted that the definition of Syndrome X as disclosed by Ferrannini et al., is not accurate. As shown by the attached **Exhibit A** (Ford and Giles, 2003), according to ATP III criteria, a patient has a metabolic syndrome if he or she has three or more of the following criteria: (1) abnormal obesity; (2) hypertriglyceridemia; (3) low HDL cholesterol; (4) high blood pressure; and (5) high fasting glucose. This publication further indicates that according to WHO criteria, a patient has a metabolic syndrome if he or she has diabetes, impaired fasting glucose, or insulin resistance plus two or more of the following: (1) high blood pressure; (2) hyperlipidemia; (3) central obesity; and (4) microalbuminuria (see page 576, left column). **Exhibit H** (National Cholesterol Education Program, 2001), indicates that diagnosis of metabolic syndrome is made when three or more of the risk determinants shown in table 8 are present (page 6, Table 8).

Taken in context, the appropriate definition of metabolic syndrome cannot be learned from Ferrannini et al. which indicates that metabolic syndrome is characterized by the concomitant occurrence of any one or more of the listed parameters. In this regard, claim 29 has been amended to indicate that syndrome X comprises more than one of the four parameters listed.

As indicated by the Examiner, a substance treating dyslipidemia may be considered by the skilled artisan as an appropriate medicament for metabolic syndrome. Garg et al., 1990, a copy of

which is attached hereto as **Exhibit I**, describe that nicotinic acid that is used as a first-line hypolpidemic drug, leads to worsening of hyperglycemia and development of hyperuricemia. The authors therefore suggest that this drug should not be used as a first-line hypolipidemic drug in patients with non-insulin-dependent diabetes mellitus. This example teaches that a hypolipidemic drug that should not be used for treating metabolic syndrome, and therefore illustrates that combination of the teaching of Ferrannini et al. with the very particular and inconsistent results of Russell et al., cannot lead the skilled artisan to conclude that MEDICA 16 may be used for treating metabolic syndrome. Moreover, Mason et al., 2005, a copy of which is attached hereto as **Exhibit J,** shows another example of a compound that is used for treating hypertension (known as one of the parameter of metabolic syndrome), that cannot be used for treating metabolic syndrome. More specifically, this publication shows that thiazide-type diuretic and beta-blocker combinations used efficiently for treating hypertension, lead to development of diabetes and therefore cannot be used for treating metabolic syndrome. Thus, a compound efficient in treatment of one parameter of metabolic syndrome (hypertension) would not necessarily be appropriate for treating metabolic syndrome. Taken together, Ferrannini et al. alone or in combination with Russell et al., cannot be considered as affecting the novelty or the inventive step of the present application.

As for the Examiner's indication that Russell et al. expressly states that the efficacy seen with MEDICA 16 in the JCR:LA-corpulent rat clearly suggests its use in the treatment of the obese, insulin-resistant, hypertriglyceridemic syndrome that is common in Western societies (i.e., human societies) and is also strongly associated with atherosclerotic disease (col.2, para.2, p.608). Applicant asserts that such statement would be considered by the skilled artisan as unsupported, since Russell et al.

clearly indicate that there was no difference in the glucose tolerance or insulin response in MEDICA 16 treated rats (Table 2, and page 605) and that "the decrease in the rate of plasma glucose clearance seen in treated male rats Is not readily explainable (page 607, right column).

In summary, Russell et al. in combination with the other cited publications, does not render obvious applicant's claimed invention.

Obviousness-Type Double Patenting

In the February 25, 2008 Office Action, the Examiner provisionally rejected claims 29-33 and 36-39 on the grounds of nonstatutory obviousness-type double patenting as unpatentable over claims 10-12 and 17 of U.S. Patent Application No.11/894,588 and/or over claims 1-3 and 8 of U.S. Patent No. 6,303,653. The Examiner also provisionally rejected claims 29-33, 36-39, 42, 44-46 and 49-52 on the grounds of nonstatutory obviousness-type double patenting as unpatentable over claims 1-4, 8-12, and 16-29 of U.S. Patent Application No.10/585,017.

Applicant's Reply

In response, applicant respectfully traverses the Examiner's rejection. However, if this provisional obviousness-type double patenting rejection is the only rejection remaining in the subject application, applicant will consider filling a terminal disclaimer M.P.E.P. § 804(I)(B).

Conclusion

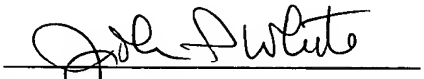
In view of the preceding amendments to the claims and the remarks set forth hereinabove, applicant respectfully submits that the grounds of rejection set forth in the February 25, 2008 Final Office Action have been overcome. Therefore, applicant

respectfully requests that the Examiner reconsider and withdraw these grounds of rejection, and solicit allowance of the claims now pending in the subject application, namely, pending claims 29-42 and 44-54 as amended.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicant's undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee, other than the enclosed $525.00 fee for a three-month extension of time is deemed necessary in connection with the filing of this Amendment. However, if any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,



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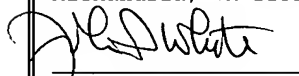

8/25/08

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EXHIBIT A

Applicants: Jacob Bar-Tana
Serial No.: 10/735,439
Filed: December 11, 2003

Exhibit A

A Comparison of the Prevalence of the Metabolic Syndrome Using Two Proposed Definitions

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WAYNE H. GILES, MD, MSC[2]

OBJECTIVE — To compare the prevalence of the metabolic syndrome using two definitions: one proposed by the Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III [ATP III]) and one by the World Health Organization (WHO).

RESEARCH DESIGN AND METHODS — We used data from a nationally representative sample of the noninstitutionalized civilian population of the U.S. from the Third National Health and Nutrition Examination Survey, a cross-sectional health examination survey (1988–1994).

RESULTS — Among 8,608 participants aged ≥20 years, the age-adjusted prevalence was 23.9% using the ATP III definition and 25.1% using the WHO definition. Among all participants, 86.2% were classified as either having or not having the metabolic syndrome under both definitions. Estimates differed substantially for some subgroups, however. For example, in African-American men, the WHO estimate was 24.9%, compared with the ATP III estimate of 16.5%.

CONCLUSIONS — A universally accepted definition of the metabolic syndrome is needed.

Diabetes Care 26:575–581, 2003

Although clustering of some metabolic abnormalities was recognized as early as 1923 (1), the coining of the term "syndrome X" in 1988 by Reaven (2) renewed the impetus to conduct research concerning this syndrome. In his description of syndrome X, Reaven considered the following abnormalities: resistance to insulin-stimulated glucose uptake, glucose intolerance, hyperinsulinemia, increased VLDL triglycerides, decreased HDL cholesterol, and hypertension. Other metabolic abnormalities that have been considered as part of the syndrome include abnormal weight or weight distribution, inflammation, microalbuminuria, hyperuricemia, and abnormalities of fibrinolysis and of coagulation (3).

People with the metabolic syndrome are at increased risk for cardiovascular disease (4) and for increased mortality from both cardiovascular disease and all causes (5). Other studies also have found that clustering of risk factors proposed to be part of the metabolic syndrome may increase the risk for coronary heart disease (6). In addition, components of the metabolic syndrome are risk factors for diabetes (7).

Because of the increased risk for morbidity and mortality associated with the metabolic syndrome, an understanding of the dimensions of this syndrome is critical both for allocating health care and research resources and for other purposes. However, generating such estimates has been complicated by the use of many definitions of the metabolic syndrome, and no standard definition has been routinely used. The World Health Organization (WHO) initially proposed a definition for the metabolic syndrome in 1998 (8). More recently, the Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III [ATP III]) provided a new working definition of the metabolic syndrome (9). Thus, prevalence estimates of the metabolic syndrome in the same population could differ depending on the definition used.

Therefore, we set out to accomplish several goals. First, to examine how prevalence estimates might differ according to the definition used, we calculated estimates of the prevalence of the metabolic syndrome by applying the ATP III and WHO definitions to data from the Third National Health and Nutrition Examination Survey (NHANES III). Second, we aimed to compare the degree to which participants were being similarly or differently classified by the two definitions. Third, little is known about how comparably the two definitions may predict the risk of future morbidity and mortality in a population. Because we were unable to examine this issue prospectively, we compared the cross-sectional associations between the prevalence of cardiovascular disease and the metabolic syndrome using both definitions.

RESEARCH DESIGN AND METHODS

— NHANES III, conducted by the National Center for Health Statistics of the Centers for Disease Control and Prevention, was started in 1988 and completed in 1994. Using a multistage, stratified sampling design, a representative sample of the civilian noninstitutionalized population consisting of 20,050

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Abbreviations: ATP III, Adult Treatment Panel III; HOMA, homeostatis model assessment; NHANES III, Third National Health and Nutrition Examination Survey; WHO, World Health Organization.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Applicants: Jacob Bar-Tana
U.S. Serial No.: 10/735,439
Filed: December 11, 2003
Exhibit A

people aged ≥17 years was recruited into the survey. After an interview in the home, participants were invited to attend one of three examination sessions: morning, afternoon, or evening. Some participants who were unable to attend the examination because of health reasons received a limited examination at home. Details about the survey and its methods have been published (10,11).

Metabolic syndrome

According to ATP III criteria (9), a participant has the metabolic syndrome if he or she has three or more of the following criteria:

1. Abdominal obesity: waist circumference >102 cm in men and >88 cm in women
2. Hypertriglyceridemia: ≥150 mg/dl (1.695 mmol/l)
3. Low HDL cholesterol: <40 mg/dl (1.036 mmol/l) in men and <50 mg/dl (1.295 mmol/l) in women
4. High blood pressure: ≥130/85 mmHg
5. High fasting glucose: ≥110 mg/dl (≥6.1 mmol/l)

According to WHO criteria (8), a participant has the metabolic syndrome if he or she has diabetes, impaired glucose tolerance, impaired fasting glucose, or insulin resistance plus two or more of the following abnormalities:

1. High blood pressure: ≥160/90 mmHg
2. Hyperlipidemia: triglyceride concentration ≥150 mg/dl (1.695 mmol/l) and/or HDL cholesterol <35 mg/dl (0.9 mmol/l) in men and <39 mg/dl (1.0 mmol/l) in women
3. Central obesity: waist-to-hip ratio of >0.90 in men or >0.85 in women and/or BMI >30 kg/m^2
4. Microalbuminuria: urinary albumin excretion rate ≥20 μg/min or an albumin-to-creatinine ratio ≥20 mg/g.

Because only fasting glucose values were available for all participants aged ≥20 years, we defined hyperglycemia for analyses involving all participants as a glucose level ≥110 mg/dl (≥6.1 mmol/l) or the current use of antidiabetic medication (insulin or oral agents). Thus, the WHO prevalence estimates for participants aged ≥20 years include patients with diabetes and impaired fasting glucose but not impaired glucose tolerance. For a second set of analyses of participants aged 40–74 years who had an oral glucose tolerance test during the morning examination, we defined diabetes, impaired glucose tolerance, and impaired fasting glucose using the baseline and 2-h glucose concentration measurements as defined by Alberti and Zimmet (8). Participants who reported using insulin did not participate in the oral glucose tolerance test, and therefore we assigned both them and participants using oral antidiabetic medications as having diabetes.

After excluding participants with self-reported diabetes or fasting blood glucose ≥126 mg/dl from our analytic sample, we defined insulin resistance as the upper quartile (≥2.68) of the distribution of the calculated homeostatis model assessment (HOMA) calculated from the following equation: $HOMA_{IR}$ = fasting insulin (μU/ml) × fasting plasma glucose (mmol/l)/22.5 (12). We used an albumin-to-creatinine ratio ≥20 mg/g because a test to determine urinary albumin excretion rate was not administered to participants.

Three readings of systolic and diastolic blood pressure were obtained from participants who attended the mobile examination center. We used the average of the last two measurements. We considered the current use of antihypertensive medication as an indication of high blood pressure. BMI was calculated from measured weight and height (weight in kilograms divided by height in meters squared). The waist circumference was measured at the high point of the iliac crest at minimal respiration to the nearest 0.1 cm. Hip circumference was measured at the maximal extension of the buttocks.

Serum glucose concentration was measured using an enzymatic reaction (Cobas Mira assay). Insulin was measured using a radioimmunoassay (insulin radioimmunoassay kit; Pharmacia Diagnostics, Uppsala, Sweden). Serum triglycerides were measured enzymatically after hydrolysis to glycerol on a Hitachi 704 analyzer (Boehringer Mannheim Diagnostics, Indianapolis, IN). HDL cholesterol was measured after precipitation of other lipoproteins with a heparin–manganese chloride mixture on a Hitachi 704 analyzer. Urinary albumin was measured using a fluorescent immunoassay on a Sequoia-Turner fluorometer (Mountain View, CA). Urinary creatinine was measured by the rate of color formation on a Beckman Synchron AS/ASTRA clinical analyzer (Beckman Instruments, Brea, CA) after creatinine reacted with picrate. Details about the laboratory procedures of all these tests are found elsewhere (11). Participants who responded affirmatively to separate questions about whether they had ever been told by a doctor that they had a heart attack, stroke, or congestive heart failure were considered to have the condition.

Pregnant women and participants who had fasted <8 h were excluded from analyses. We performed a set of analyses on a sample of participants aged 40–74 years who had an oral glucose tolerance test. We calculated the prevalence of the metabolic syndrome by age, sex, and race or ethnicity (white, African-American, Mexican-American, and other). Age adjustment was performed using the age distribution of the U.S. population in the year 2000. Because of the complex sampling design, all analyses were performed using software for the statistical analysis of correlated data (SUDAAN) to obtain proper variance estimates (13).

RESULTS — A total of 8,608 participants aged ≥20 years had complete information for the study variables and were included in the analyses. They included 4,167 men, 4,441 women, 3,500 whites, 2,372 African-Americans, 2,388 Mexican-Americans, and 348 participants of other races or ethnicities. The age-adjusted prevalences of the individual criteria of the metabolic syndrome are listed in Table 1. The high prevalence of central adiposity as defined by WHO was largely driven by the fact that 72.3% (unadjusted) of men had a waist-to-hip ratio >0.90, and 49.6% (unadjusted) of the women had a waist-to-hip ratio >0.85.

We classified 23.9 and 25.1% of the participants as having the metabolic syndrome using the ATP III definition and the WHO definition, respectively (Table 2). Among all participants, 86.2% were similarly classified under the two definitions. Under the ATP III definition but not the WHO definition, 6.2% of participants had the metabolic syndrome, and 7.6% of participants had the metabolic syndrome under the WHO definition but not the ATP III definition. Despite the similar estimates for the entire sample, substantial differences were noted for some subgroups. WHO estimates were similar to the ATP III estimates among whites but were higher for the other race or ethnic groups. The largest difference occurred

among African-American men, of whom 16.5% had the metabolic syndrome using ATP III criteria and 24.9% had the metabolic syndrome using WHO criteria.

Among the participants who were classified as having the metabolic syndrome using the ATP III criteria but not the WHO criteria, 89.0% met at least two of the four WHO criteria but did not have hyperglycemia and were not insulin resistant. Conversely, among the participants who were classified as having the metabolic syndrome using the WHO criteria but not the ATP III criteria, 82.4% had two of the ATP III criteria.

The age-adjusted serum insulin concentrations (mean ± SE) were 104.8 ± 3.5 pmol/l for 2,217 participants who met the ATP III definition of the metabolic syndrome and 50.9 ± 0.8 pmol/l for the 6,391 participants without the metabolic syndrome ($P < 0.001$). The mean age-adjusted HOMA was 4.95 ± 0.16 for participants who met the ATP III definition of the metabolic syndrome and 2.00 ± 0.03 for those without the metabolic syndrome ($P < 0.001$). The age-adjusted proportion of participants with HOMA in the top quintile was 64.1 ± 1.8% for participants who met the ATP III definition of the metabolic syndrome and 12.9 ± 0.7% for those without the metabolic syndrome ($P < 0.001$).

The age-adjusted mean serum insulin concentrations were 113.2 ± 3.1 pmol/l for 2,536 participants who met the WHO definition of the metabolic syndrome and 46.5 ± 0.6 pmol/l for the 6,072 participants without the metabolic syndrome ($P < 0.001$). The mean age-adjusted HOMA was 5.30 ± 0.15 for participants who met the ATP III definition of the metabolic syndrome and 1.81 ± 0.02 for those without the metabolic syndrome ($P < 0.001$). The age-adjusted proportion of participants with HOMA in the top quintile was 81.3 ± 1.6% for participants who met the ATP III definition of the metabolic syndrome and 5.9 ± 0.5% for those without the metabolic syndrome ($P < 0.001$).

Oral glucose tolerance test sample (participants aged 40–74 years)

A total of 2,857 participants were included in these analyses. The age-adjusted prevalences of the metabolic syndrome were 33.9 and 36.9% for the definitions from ATP III and WHO, respectively (Table 3). Of the participants,

Table 1—*Age-adjusted prevalence of individual metabolic abnormalities of the metabolic syndrome as defined by ATP III and WHO among 8,608 U.S. adults aged ≥20 years (NHANES III, 1988–1994)*

	ATP III						WHO					
	Abdominal obesity	Hypertriglyceridemia	Low HDL cholesterol	High blood pressure or medication use	High glucose or medication use	Central obesity	Hyperlipidemia	High blood pressure or medication use	Glucose >110 mg/dl or medication use	Insulin resistance	Albumin-to-creatinine ratio ≥20 mg/g
Total	38.7 ± 0.9	29.8 ± 1.1	37.0 ± 1.3	34.0 ± 0.8	12.6 ± 0.5	67.5 ± 0.9	48.6 ± 1.4	19.0 ± 0.7	12.6 ± 0.5	26.3 ± 0.9	12.6 ± 0.5
Men	30.4 ± 1.2	35.0 ± 1.7	35.1 ± 1.5	38.2 ± 1.4	15.6 ± 0.8	77.3 ± 1.1	49.5 ± 1.7	19.6 ± 1.1	15.6 ± 0.8	28.6 ± 1.3	11.1 ± 0.7
Women	46.7 ± 1.2	24.6 ± 1.0	39.1 ± 1.5	29.4 ± 0.8	9.9 ± 0.6	57.8 ± 1.3	47.8 ± 1.6	18.0 ± 0.8	9.9 ± 0.6	24.2 ± 1.2	14.1 ± 0.7
Race or ethnicity											
White	37.8 ± 0.9	30.9 ± 1.3	37.7 ± 1.6	32.8 ± 1.0	11.9 ± 0.6	66.0 ± 1.1	49.4 ± 1.6	18.2 ± 0.8	11.9 ± 0.6	23.9 ± 1.1	11.6 ± 0.6
African-American	44.8 ± 1.2	17.8 ± 0.8	28.7 ± 1.4	46.6 ± 0.9	15.2 ± 0.9	67.9 ± 0.7	37.1 ± 1.3	29.9 ± 0.9	15.2 ± 0.9	34.5 ± 1.5	17.7 ± 0.9
Mexican-American	45.5 ± 1.3	38.2 ± 1.0	39.9 ± 1.4	36.7 ± 1.2	20.0 ± 1.0	82.9 ± 0.8	56.3 ± 1.3	17.8 ± 1.0	20.0 ± 1.0	40.1 ± 1.0	15.7 ± 1.1
Other	33.8 ± 5.3	27.2 ± 3.3	37.1 ± 4.5	29.7 ± 2.9	14.1 ± 2.0	68.9 ± 2.8	48.0 ± 4.0	13.7 ± 2.1	14.1 ± 2.0	30.2 ± 3.5	14.7 ± 2.1
Men											
White	31.3 ± 1.2	36.8 ± 2.0	36.6 ± 1.7	37.3 ± 1.7	15.6 ± 1.0	78.4 ± 1.3	51.2 ± 1.8	18.8 ± 1.4	15.6 ± 1.0	27.7 ± 1.5	9.9 ± 0.8
African-American	23.5 ± 1.3	21.3 ± 1.2	22.6 ± 1.8	49.6 ± 1.6	14.5 ± 1.1	65.3 ± 1.3	34.0 ± 1.5	30.2 ± 1.3	14.5 ± 1.1	29.3 ± 1.8	18.5 ± 1.3
Mexican-American	30.0 ± 1.9	40.2 ± 1.5	34.1 ± 2.2	39.8 ± 1.8	21.1 ± 1.4	86.7 ± 1.0	52.9 ± 1.8	20.2 ± 1.3	21.1 ± 1.4	37.8 ± 2.0	13.4 ± 1.6
Other	26.6 ± 7.5	29.1 ± 4.0	33.2 ± 5.2	34.3 ± 4.0	14.9 ± 3.4	72.6 ± 4.4	47.0 ± 4.5	16.1 ± 3.2	14.9 ± 3.4	30.1 ± 4.4	13.1 ± 2.9
Women											
White	43.8 ± 1.4	24.8 ± 1.1	39.1 ± 1.9	27.8 ± 0.9	8.4 ± 0.6	53.4 ± 1.5	47.8 ± 2.1	17.1 ± 0.9	8.4 ± 0.6	20.3 ± 1.3	13.5 ± 0.8
African-American	62.3 ± 1.6	14.7 ± 1.0	33.9 ± 1.7	43.8 ± 1.3	15.7 ± 1.4	70.0 ± 1.2	39.7 ± 1.7	29.6 ± 1.3	15.7 ± 1.4	38.7 ± 2.3	17.1 ± 1.0
Mexican-American	63.2 ± 1.8	35.8 ± 1.5	46.6 ± 1.6	32.9 ± 1.2	18.9 ± 1.3	78.6 ± 1.1	60.3 ± 1.5	15.1 ± 1.1	18.9 ± 1.3	42.9 ± 1.7	18.5 ± 1.5
Other	40.4 ± 4.8	26.0 ± 4.4	39.8 ± 4.6	23.8 ± 2.3	14.4 ± 2.9	65.8 ± 5.1	46.5 ± 4.4	10.9 ± 2.3	14.4 ± 2.9	31.0 ± 4.8	16.1 ± 3.5

Data are % ± SE.

Table 2—*Prevalence of the metabolic syndrome using the ATP III and WHO criteria among 8,608 U.S. adults aged ≥20 years (NHANES III, 1988–1994)*

	Age-adjusted			Unadjusted	
	ATP III	WHO	Agreement*	ATP III = yes, WHO = no	WHO = yes, ATP III = no
Total	23.9 ± 0.8	25.1 ± 0.9	86.2 ± 0.7	6.2 ± 0.3	7.6 ± 0.6
Men	24.2 ± 1.2	27.9 ± 1.1	86.1 ± 0.8	5.2 ± 0.4	8.8 ± 0.7
Women	23.5 ± 0.9	22.6 ± 1.1	86.3 ± 0.8	7.2 ± 0.4	6.5 ± 0.7
Race or ethnicity					
White	24.0 ± 1.0	23.8 ± 1.0	86.5 ± 0.8	6.8 ± 0.4	6.7 ± 0.6
African-American	21.9 ± 0.9	28.0 ± 1.2	86.0 ± 1.0	3.8 ± 0.4	10.2 ± 0.8
Mexican-American	32.0 ± 1.4	38.1 ± 1.1	84.2 ± 0.7	4.3 ± 0.5	11.5 ± 0.7
Other	20.3 ± 3.4	26.5 ± 3.0	84.6 ± 2.9	4.7 ± 1.8	10.7 ± 2.5
Men					
White	25.1 ± 1.5	27.6 ± 1.2	86.4 ± 1.0	5.6 ± 0.5	8.0 ± 0.9
African-American	16.5 ± 1.0	24.9 ± 1.3	88.0 ± 1.1	2.1 ± 0.4	9.9 ± 1.0
Mexican-American	28.0 ± 1.9	36.0 ± 1.6	84.5 ± 0.7	3.8 ± 0.6	11.7 ± 0.9
Other	20.8 ± 4.8	28.3 ± 3.6	82.4 ± 3.8	5.3 ± 2.3	12.3 ± 3.0
Women					
White	22.7 ± 1.1	20.3 ± 1.2	86.7 ± 0.9	7.9 ± 0.6	5.4 ± 0.7
African-American	26.1 ± 1.3	30.5 ± 1.8	84.4 ± 1.3	5.2 ± 0.6	10.5 ± 1.2
Mexican-American	36.3 ± 1.5	40.5 ± 1.4	83.8 ± 1.3	4.9 ± 0.7	11.3 ± 1.2
Other	19.9 ± 3.1	24.8 ± 3.3	86.9 ± 3.1	4.1 ± 1.7	9.0 ± 2.8

Data are % ± SE except the difference. *Percent of participants who were classified as either having or not having the metabolic syndrome under both definitions of the metabolic syndrome.

81.9% were similarly classified under either of the two definitions. Under the ATP III definition but not the WHO definition, 7.6% of the participants had the metabolic syndrome, and 10.5% had the metabolic syndrome under the WHO definition but not the ATP III definition.

The age-adjusted mean serum insulin concentrations were 100.3 ± 3.2 pmol/l for 1,036 participants who met the ATP III definition of the metabolic syndrome and 53.0 ± 1.2 pmol/l for 1,821 partici-

Table 3—*Prevalence of the metabolic syndrome using the ATP III and WHO criteria among 2,857 U.S. adults aged 40–74 years who had an oral glucose tolerance test (NHANES III, 1988–1994)*

	Age-adjusted			Unadjusted	
	ATP III	WHO	Agreement*	ATP III = yes, WHO = no	WHO = yes, ATP III = no
Total	33.9 ± 1.5	36.9 ± 1.5	81.9 ± 1.1	7.6 ± 0.6	10.5 ± 0.9
Men	34.8 ± 2.0	41.3 ± 2.3	80.9 ± 1.7	6.4 ± 1.1	12.8 ± 1.3
Women	33.0 ± 1.9	32.7 ± 1.8	82.9 ± 1.3	8.8 ± 0.7	8.4 ± 1.1
Race or ethnicity					
White	34.6 ± 1.8	35.9 ± 1.6	82.2 ± 1.2	8.4 ± 0.8	9.4 ± 0.9
African-American	29.5 ± 1.9	37.5 ± 2.2	80.0 ± 1.4	6.1 ± 0.9	13.9 ± 1.1
Mexican-American	45.5 ± 2.0	53.0 ± 2.0	78.8 ± 2.3	7.0 ± 1.3	14.2 ± 1.7
Other	24.1 ± 4.9	35.9 ± 4.7	81.8 ± 4.4	1.4 ± 0.8	16.9 ± 4.5
Men					
White	36.4 ± 2.5	41.0 ± 2.4	81.6 ± 1.8	7.2 ± 1.3	11.2 ± 1.4
African-American	21.6 ± 2.6	35.3 ± 2.9	77.9 ± 2.0	4.1 ± 1.3	18.0 ± 1.6
Mexican-American	39.4 ± 2.8	48.4 ± 2.6	80.6 ± 3.3	5.9 ± 1.7	13.5 ± 2.6
Other	27.8 ± 6.9	43.7 ± 6.0	76.3 ± 7.1	0.6 ± 0.6	23.1 ± 7.1
Women					
White	32.9 ± 2.1	31.2 ± 2.0	82.9 ± 1.5	9.5 ± 0.8	7.6 ± 1.2
African-American	35.8 ± 2.9	39.4 ± 3.3	81.6 ± 1.8	7.6 ± 1.2	10.8 ± 1.5
Mexican-American	51.9 ± 2.8	58.3 ± 3.5	76.9 ± 2.8	8.2 ± 1.7	15.0 ± 2.1
Other	21.4 ± 6.0	28.2 ± 5.6	87.3 ± 4.4	2.1 ± 1.7	10.6 ± 5.0

Data are % ± SE except the difference. *Percent of participants who were classified as either having or not having the metabolic syndrome under both definitions of the metabolic syndrome.

Table 4—*Prevalence of self-reported heart attack, stroke, and congestive heart failure by metabolic syndrome status defined by the ATP III and WHO among U.S. adults aged ≥20 years (NHANES III, 1988–1994)*

	Metabolic syndrome		No metabolic syndrome				
	Sample size	Age-adjusted prevalence	Sample size	Age-adjusted prevalence	*P*	Sample size	Odds ratio*
ATP III							
Heart attack	2,209	4.5 ± 0.6	6,313	2.9 ± 0.3	0.017	8,372	1.59 (1.12–2.25)
Stroke	2,216	3.0 ± 0.6	6,389	1.3 ± 0.2	0.008	8,455	2.39 (1.40–4.09)
Congestive heart failure	2,210	3.1 ± 0.6	6,387	1.8 ± 0.3	0.056	8,446	1.81 (1.06–3.10)
WHO							
Heart attack	2,515	5.1 ± 0.6	6,007	2.6 ± 0.3	<0.001	8,372	2.03 (1.41–2.91)
Stroke	2,535	2.8 ± 0.4	6,070	1.3 ± 0.2	<0.001	8,455	2.17 (1.47–3.22)
Congestive heart failure	2,528	3.6 ± 0.6	6,069	1.5 ± 0.2	0.002	8,446	2.53 (1.50–4.26)

Data are *n*, % ± SE, or OR (95% CI). *Odds ratio is adjusted for age, sex, race or ethnicity, education, smoking status, cotinine concentration, and non-HDL cholesterol concentration.

pants without the metabolic syndrome ($P < 0.001$). The mean age-adjusted HOMA was 5.16 ± 0.21 for participants who met the ATP III definition of the metabolic syndrome and 2.15 ± 0.05 for those without the metabolic syndrome ($P < 0.001$). The age-adjusted proportion of participants with HOMA in the top quintile was 68.4 ± 2.1% for participants who met the ATP III definition of the metabolic syndrome and 15.8 ± 1.5% for those without the metabolic syndrome ($P < 0.001$).

The age-adjusted mean serum insulin concentrations were 103.3 ± 3.1 pmol/l for 1,180 participants who met the WHO definition of the metabolic syndrome and 49.3 ± 0.8 pmol/l for 1,677 participants without the metabolic syndrome ($P < 0.001$). The mean age-adjusted HOMA was 5.22 ± 0.19 for participants who met the ATP III definition of the metabolic syndrome and 1.97 ± 0.04 for those without the metabolic syndrome ($P < 0.001$). The age adjusted proportion of participants with HOMA in the top quintile was 78.2 ± 1.6% for participants who met the ATP III definition of the metabolic syndrome and 8.1 ± 0.9% for those without the metabolic syndrome ($P < 0.001$).

Prevalence of self-reported heart attack, stroke, and congestive heart failure

The prevalence of heart attack was 4.5% among participants with the ATP III–defined syndrome, 2.9% among those without the ATP III–defined syndrome, 5.1% among those with the WHO-defined syndrome, and 2.6% among those without the WHO-defined syndrome (Table 4). However, the confidence intervals of the corresponding ATP III and WHO estimates overlap considerably, suggesting that the prevalence of cardiovascular disease is similar. Although the adjusted odds ratios for heart attack and congestive heart failure were higher when we used the WHO definition, the confidence intervals of the odds ratios overlap considerably. However, prevalence estimates for stroke were similar for participants with the metabolic syndrome defined by either definition.

CONCLUSIONS — Under either definition of the metabolic syndrome, its prevalence in the U.S. population is common. We previously reported that ~22% of U.S. adults have the metabolic syndrome according to ATP III criteria (14). The prevalence estimate reported here differs slightly from our earlier estimate because of differences in the analytic sample sizes. Although the two definitions yield similar prevalence estimates for the entire sample (despite considerable differences in the two definitions), the two estimates differed markedly for various population subgroups, especially for some race or ethnic groups. Of the participants, ~80–85% would be classified as having or not having the metabolic syndrome under either definition, suggesting that the two definitions are identifying a similar group of people. However, ~15–20% of participants are classified differently under the two approaches, with roughly half being classified as having the metabolic syndrome under one definition and the other half under the other definition.

That the two definitions classify large numbers of participants as having the metabolic syndrome is perhaps not too surprising, given that the two definitions use many of the same variables: central or abdominal adiposity, dyslipidemia, hypertension, and hyperglycemia. By including insulin resistance explicitly, the WHO definition identifies participants with the metabolic syndrome more directly. In contrast, the ATP III definition does not include a direct measure to identify insulin-resistant people. However, the five ATP III criteria are to some degree associated with insulin resistance. Thus, these criteria may result indirectly in the identification of many participants who are likely to have insulin resistance. The reasons why there is not better agreement are more difficult to discern. However, 80–90% of the participants who are classified as having the metabolic syndrome using one definition but not the other fail to meet one additional criterium that would cause them to meet both definitions of the metabolic syndrome.

The WHO criteria for central obesity appear to account for much of the higher prevalence of WHO-defined metabolic syndrome. The prevalence of central obesity as defined by the WHO among African-American men is about three times higher than the ATP III prevalence of abdominal obesity, compared with the approximate twofold difference in these two measures among the other three groups of men. When we substituted the ATP III abdominal obesity criteria for the WHO criteria, the prevalence of WHO-defined metabolic syndrome for the entire sample dropped to 21.3% from 25.1%. Among African-American men, the prev-

alence decreased to 18.3% from 24.9%. In addition, the higher prevalence of microalbuminuria among nonwhites compared with white participants partially explained the higher prevalence of WHO-defined metabolic syndrome. Among men, African-American men had the highest prevalence of microalbuminuria, whereas among women, Mexican-American women had the highest prevalence. When we recalculated the WHO prevalences without the criteria for microalbuminuria, the prevalences decreased in all groups. For example, the prevalence for African-American men decreased to 23.3% from 24.9%.

Neither definition explicitly includes the use of medications for hypertension, glucose intolerance, or dyslipidemia as part of the definition. We chose to include medications for hypertension and glucose intolerance. We did not do so for dyslipidemia because no specific questions were asked of participants about their use of medications for this purpose. Participants were asked only about cholesterol-lowering medications. We recognize that some of these medications may also lower triglycerides or elevate HDL cholesterol concentrations. To the degree that these medications do so, the ATP III and WHO prevalence estimates would have been underestimated. The criteria for hypertension differ significantly under the two definitions, with the ATP III report advocating the use of 130/85 mmHg and the WHO definition 160/95 mmHg. By including antihypertensive medication use in the definition, our estimates for hypertension using the WHO definition may have been slightly inflated because medication for hypertension probably was prescribed for participants with blood pressure levels between the two thresholds. Consequently, the prevalence estimate for the metabolic syndrome may also have been slightly inflated. Thus, the net effect of these two sources of misclassification may be that the ATP III estimate was slightly underestimated, whereas the two sources of misclassification that affected the WHO prevalence may have cancelled each other to a certain extent.

An important consideration in estimating the prevalence of the metabolic syndrome using the WHO definition is how to operationalize insulin resistance. Different authors have defined insulin resistance using surrogate measures (fasting insulin concentration, HOMA, etc.) in a myriad of ways. A key consideration is how to establish a cut point for these measures, because the choice of a cut point will affect the prevalence estimates of the metabolic syndrome. Typically, this has been done by using some percentile of the distribution of a surrogate measure. Initial inclusion and exclusion criteria at the time of recruitment of study participants and additional inclusion or exclusion criteria applied to the study participants after their recruitment define a final subset of study participants who are used to produce thresholds for insulin resistance.

In an attempt to examine which definition of the metabolic syndrome might be more strongly associated with the risk for cardiovascular disease, we compared the prevalences of self-reported heart attack, stroke, and congestive heart failure for the two definitions of the metabolic syndrome. For self-reported heart attack and congestive heart failure, the WHO definition yielded higher prevalence estimates and odds ratios than those from the ATP III definition, although the confidence intervals were not mutually exclusive. By making insulin resistance one of its criteria, the WHO definition may be including more people with insulin resistance or people who are more insulin resistant than the ATP III definition would include. Findings that insulin resistance is associated with an increased risk for cardiovascular disease events (15,16) could help to explain the slightly higher prevalence estimates of cardiovascular disease among participants with the metabolic syndrome as defined by the WHO. In addition, some evidence suggests that microalbuminuria may be associated with higher relative risks for fatal and nonfatal cardiovascular disease than other components of the metabolic syndrome (4,17). Recent reviews support the notion that microalbumiuria may predict the risk for cardiovascular disease in diabetic and nondiabetic populations, although additional study of this association is still needed (18,19). If microalbuminuria does indeed predict cardiovascular disease risk, this may possibly help to explain why the WHO definition yielded higher prevalence estimates of heart attack and congestive heart failure and higher odds ratios than the ATP III definition. Furthermore, the higher threshold for hypertension used by the WHO definition also may have led to selective enrichment of participants with the metabolic syndrome who are at increased risk for heart disease. However, the stroke prevalence was similar for both definitions. Prospective studies are needed to corroborate these cross-sectional findings and better estimate the risks associated with each definition of the metabolic syndrome.

In conclusion, the prevalence of the metabolic syndrome is common using either the ATP III definition or the proposed WHO definition. Furthermore, both definitions yielded similar estimates for the entire population but masked underlying differences for various population subgroups. However, for clinical, epidemiological, and surveillance purposes, a unified definition may be desirable. Whether clinical implications exist for the 15–20% of participants who would be differently defined by the two definitions is of some concern. Clearly, this group has a number of abnormalities and are likely to benefit from weight control or increases in physical activity.

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EXHIBIT B

Applicants: Jacob Bar-Tana
Serial No.: 10/735,439
Filed: December 11, 2003

Exhibit B (1c)

Inhibition of Atherosclerosis and Myocardial Lesions in the JCR:LA-cp Rat by β,β'-Tetramethylhexadecanedioic Acid (MEDICA 16)

James C. Russell, Roger M. Amy, Sandra E. Graham, Peter J. Dolphin, George O. Wood, Jacob Bar-Tana

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Inhibition of Atherosclerosis and Myocardial Lesions in the JCR:LA-cp Rat by β,β′-Tetramethylhexadecanedioic Acid (MEDICA 16)

James C. Russell, Roger M. Amy, Sandra E. Graham, Peter J. Dolphin, George O. Wood, Jacob Bar-Tana

Abstract Atherosclerosis-prone, insulin-resistant JCR:LA-cp male rats were treated from 6 weeks to 39 weeks of age with β,β′-tetramethylhexadecanedioic acid (MEDICA 16). Body weights were reduced (13%, $P<.001$) at 36 weeks without any accompanying decrease in food consumption. The treatment did not cause any significant change in plasma glucose or fasting insulin concentrations. There was a significant decrease in the extreme hyperplasia of the islets of Langerhans (38%, $P<.05$). The marked VLDL hypertriglyceridemia was decreased by 70% ($P<.001$), with an accompanying significant reduction in cholesterol concentrations. The severity of raised atherosclerotic lesions on the aortic arch was very markedly reduced ($P<.01$) in treated rats. This was accompanied by a reduction ($P<.01$) in the incidence of ischemic myocardial lesions. We conclude that long-term (33 weeks) MEDICA 16 treatment of an animal model for the obesity/insulin-resistant/hyperlipidemic syndrome not only markedly improved lipid metabolism, but also inhibited the development of advanced cardiovascular disease. (*Arterioscler Thromb Vasc Biol.* 1995;15:918-923.)

Key Words • MEDICA 16 • myocardial lesions • hypertriglyceridemia • JCR:LA-cp rat • atherosclerosis

The JCR:LA-cp rat is one of a number of strains incorporating the autosomal recessive *cp* gene originally isolated by Koletsky.[1,2] The rats are, if homozygous normal (+/+) or heterozygous (+/*cp*), lean and indistinguishable from the parent LA/N strain. If homozygous *cp* (*cp/cp*), the rats are obese from an early age, insulin resistant, and hyperinsulinemic and exhibit a marked hyperlipidemia due to hepatic hypersecretion of VLDL.[3-5] This strain is the only one incorporating the *cp* gene to exhibit spontaneous atherosclerosis and ischemic myocardial lesions.[6-9] The atherosclerosis and myocardial lesions are essentially confined to the *cp/cp* male rats, with lean rats of both sexes and *cp/cp* females being spared.[8,9] Although the fatty Zucker (*fa*) gene and the *cp* gene are allelic,[10,11] they are clearly different in effect. In particular, the *fa/fa* (fatty) Zucker rat does not develop as severe a metabolic disturbance as does the JCR:LA-cp rat, nor does the former exhibit any frank cardiovascular disease.[8,12]

β,β′-Tetramethylhexadecanedioic acid (MEDICA 16) is the most effective of a series of long-chain fatty acids developed as hypolipidemic and antiobesity/anti–insulin resistance agents.[13] The hypolipidemic effect of MEDICA 16[14] can be accounted for by the inhibition of long-chain fatty acid and cholesterol synthesis,[15,16] together with the activation of triglyceride-rich plasma lipoprotein clearance mediated by a decrease in plasma apo C-III.[17,18] The antiobesity effect may be accounted for by increased lipolysis complemented by liver calorigenesis due to the thyromimetic activity of the drug.[19-21] The antidiabetogenic effect is accounted for by the adipose-reductive effect of the drug and by a concomitant decrease in insulin resistance.[22]

Short-term (14 days) treatment of *cp/cp* rats of the JCR:LA-cp strain with MEDICA 16 resulted in a marked decrease (up to 80%) in plasma triglycerides.[23] This was shown to be due to a decreased rate of VLDL production by the liver, secondary to inhibition of fatty acid synthesis at the level of ATP citrate lyase. There was also evidence of an increased rate of VLDL catabolism. Despite the lack of improvement in insulin and glucose metabolism, the changes in lipid metabolism were sufficiently great to suggest that MEDICA 16 might be expected to protect against the development of vascular and myocardial damage in this animal model. We report here that long-term treatment with MEDICA 16 does offer such protection.

Methods

Animals

Male rats were bred in our established colony of JCR:LA-cp rats, both +/+ and *cp/cp*, as previously described.[6,7] They were weaned at 3 weeks of age and maintained in polycarbonate cages on wood chip bedding and exposed to a 12/12-hour light/dark cycle. The room was maintained at 20°C and 55% relative humidity. Food, available at all times, was Wayne Rodent Blox (Harlan Sprague Dawley Inc), a grain-based diet containing less than 4% total fat.[24] The food was obtained in powdered form, and MEDICA 16, synthesized as previously

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described,[15] was incorporated at 0.25% (wt/wt) for the treated rats. The food, both control and treated, was moistened with water, extruded into pellet form, and air dried. This diet was fed to the rats from 6 weeks of age. The rats were weighed on a regular basis and food consumption was recorded. Otherwise the animals were left undisturbed until 39 weeks of age.

The rats were starved overnight at 39 weeks of age and anesthetized with halothane in oxygen. Blood was sampled from the left ventricle of the heart, and the rat was then perfusion-fixed with 1.25% glutaraldehyde and 1.85% formaldehyde in Tyrode's solution at 100 mm Hg. After their removal, the heart, liver, duodenum, kidneys, spleen, adrenals, testes, lungs, and brain were further fixed in neutral buffered formalin, the pancreas was fixed in Bouin's solution and 70% ethanol, and the aortic arch was fixed in 2.5% glutaraldehyde. The tissues fixed in formalin and the pancreas were processed by conventional histological techniques, sectioned, and stained with hematoxylin and eosin. The hearts were cut transversely into three blocks: apex, midheart, and base. Adjacent sections were taken from each block of the heart and stained with hematoxylin and eosin as well as Masson's trichrome stain. The heart sections were examined by an experienced pathologist who was not aware of the group to which each rat belonged, and myocardial lesions were identified and their frequencies determined as described previously.[9] Lesion stages were classified as follows: stage 1, areas of necrosis; stage 2, areas of cell lysis with chronic inflammatory cell infiltration; stage 3, nodules of chronic inflammatory cell infiltration; and stage 4, old scarred lesions. The pancreatic sections were examined histologically, and the cross-sectional areas of the islets of Langerhans and their volume densities were determined by use of an image analysis system (GENIAS25, Joyce-Loebl Div, Vickers Plc). Measurement was based on five random fields at ×10 magnification from a section of the tail of the pancreas. The aortic arch was dissected free of all extraneous tissue and split along the greater and lesser curves. The two halves of the arch, including the stumps of the branches, were postfixed with osmium tetroxide and uranyl acetate, dried with graded ethanol solutions, and triple-point dried from propylene oxide. The mounted segments were sputter-coated with gold, and the intimal surfaces were examined completely by use of a Hitachi scanning electron microscope (model S2500). Lesions were identified and classified as areas of adherent fibrin, raised intimal lesions, areas of adherent macrophages, or areas of de-endothelialization. All lesions for each animal were recorded photographically, and each type was assigned a severity score. The scale used had a range of 0 to 3, with 0 representing the absence of any lesions and 3 representing the most severe involvement.

Plasma glucose was measured by use of a rapid glucose oxidase method (Beckman Instruments). Insulin was assayed by use of a double antibody radioimmunoassay technique (Kabi Pharmacia Diagnostics AB) with rat insulin standards. Serum lipid concentrations were determined by the gas chromatographic total lipid profile technique of Kuksis et al.[25] Statistical analysis was by ANOVA and Wilcoxon's rank sum test, as appropriate, with a value of $P<.05$ for the two-tailed test taken as significant.[26]

All care and treatment of the rats were in conformity with the Guidelines of the Canadian Council on Animal Care and subject to prior institutional review and approval.

Results

Food consumption data for the *cp/cp* rats, both MEDICA 16 treated and control, were essentially identical, as shown in Fig 1. Lean control rats (+/+) ate significantly less throughout the experimental period. Body weights of the MEDICA 16–treated *cp/cp* rats were consistently lower than those of the control rats, although the former remained much greater than those of the +/+ control animals (Fig 1). The lower body weights of the treated rats became significant at 12 weeks of age ($P<.05$) and remained highly significant thereafter ($P<.001$). As shown in Table 1, there was no significant change in fasting plasma glucose, whereas insulin concentrations were highly variable, making the even quite substantial differences apparently induced by treatment nonsignificant. However, there was a significant (38%, $P<.01$) decrease in volume density of the islets of Langerhans. The pancreases of the MEDICA 16–treated rats showed abnormal islet structure and evidence of pancreatitis similar to those seen in the *cp/cp* control animals, although less severe, as illustrated in Fig 2. The pancreases of MEDICA 16–treated rats also contained frequent smaller, essentially normal islets, as shown in Fig 2D.

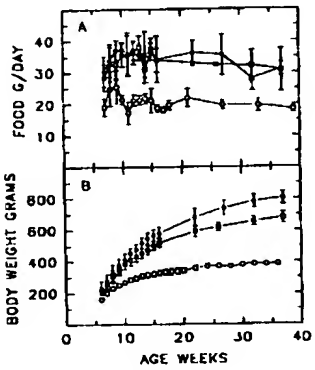

FIG 1. Food consumption (A) and body weight (B) of male rats. Values are mean±SD for 10 rats in each group. ○ indicates +/+ control; ●, *cp/cp* control; □, *cp/cp* β,β'-tetramethylhexadecanedioic acid–treated.

Table 2 shows the serum lipid concentrations in the 39-week-old rats. The long-term MEDICA 16 treatment resulted in a 70% reduction in triglycerides and significant decreases in total cholesterol (approximately 30%) and phospholipids (38%).

Scanning electron photomicrographs of the aorta, illustrating typical raised lesions with severity scores of 0 to 3, are shown in Fig 3. A score of 0 represents a smooth endothelial surface throughout, and a score of 3 represents a large raised lesion with abnormal overlying endothelium and desquamation. The extent of adhesion of macrophages to the endothelium was similarly assessed, and examples of moderate involvement (score of 2) and extensive involvement (score of 3) are shown in Fig 4A and 4B, respectively. As shown in Table 3, MEDICA 16 treatment of the *cp/cp* rats resulted in a

TABLE 1. Plasma Glucose, Insulin Concentrations, and Islet of Langerhans Volume Density in β,β'-Tetramethylhexadecanedioic Acid–Treated Male Rats

	Plasma Glucose, mg/dL	Plasma Insulin, mU/L	Islet Volume Density, %
+/+ control	128±11.0	29±15	1.77±0.66
cp/cp control	141±11.0	254±279	9.77±3.72
cp/cp MEDICA 16–treated	173±34.0	210±106	5.51±2.03*

MEDICA 16 indicates β,β'-tetramethylhexadecanedioic acid.
Values are mean±SD for ten 39-week-old rats in each group.
*$P<.01$ vs *cp/cp* control.

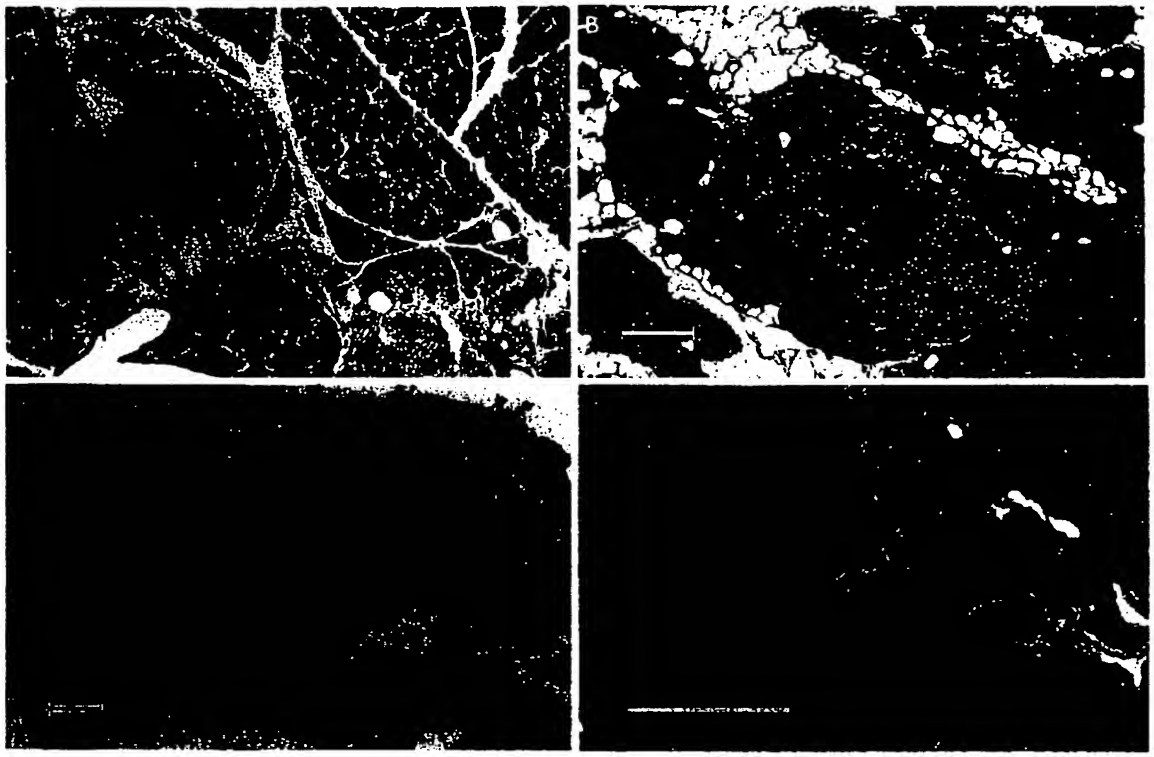

FIG 2. Photomicrographs of pancreatic sections from 9-month-old male JCR:LA-cp rats show normal islet of Langerhans from a +/+ control rat (A); highly hyperplastic islets from a *cp/cp* control rat (B); large islets and pancreatitis in a *cp/cp* β,β'-tetramethylhexadecanedioic acid–treated rat (C); and several small, essentially normal islets and one moderately enlarged islet from a treated rat (D). Sections were stained with hematoxylin and eosin. Bars indicate 200 μm.

marked and very significant reduction in the severity of atherosclerotic raised lesions on the aortic arch ($P<.01$). The reduction seen in the incidence of adherent macrophages was also substantial and significant ($P<.05$). The extent of de-endothelialization was apparently reduced, but this was not statistically significant. As shown in Table 4, the frequency of old, mature myocardial lesions (stage 4) was also markedly and significantly reduced ($P<.01$) in the MEDICA 16–treated rats. Photomicrographs of representative lesions are shown in Fig 5, illustrating the decreased size of lesions in the MEDICA 16–treated rats.

Detailed examination of the histological sections of the liver, duodenum, kidneys, spleen, adrenals, testes, lungs, and brain of the 39-week-old rats revealed no significant abnormalities in any of the animals.

Discussion

Long-term treatment of *cp/cp* male rats with MEDICA 16 resulted in a hypolipidemic effect similar to that we previously reported after short-term (2 weeks) treatment.[23] However, while short-term treatment with MEDICA 16 caused no significant change in body weight, the long-term–treated rats had significantly lower body weights despite unchanged food intake. The reduced rate of weight gain and lower body weight, together with the very marked reduction in serum VLDL concentration, must therefore reflect a significant redirection of diet-derived glucose from triglyceride synthesis. The reduced flow of triglyceride in VLDL should reduce adipocyte accumulation of lipid, and thus of body weight, as observed. Such changes must be balanced by increased glucose oxidation elsewhere, as food consumption was not significantly reduced. The highly variable fasting insulin levels that are characteristic of the *cp/cp* rat may have obscured a real increase in insulin sensitivity. The substantial decrease in islet volume density and the presence of small, essentially normal, islets in the MEDICA 16–treated rats suggest that real changes in the insulin metabolism occurred. An ex-

TABLE 2. Serum Lipid Concentrations in β,β'-Tetramethylhexadecanedioic Acid–Treated Male Rats

	Cholesterol, mg/dL	Cholesteryl Esters, mg/dL	Phospholipids, mg/dL	Triglycerides, mg/dL	Total Cholesterol, mg/dL
+/+ control	11.2±16	61±11	55±11	19.9±8.1	46.7±7.8
cp/cp control	36.1±11.5	181±60	220±69	309±101	142±46
cp/cp MEDICA 16–treated	27.7±4.5*	117±28.3†	145±32.7†	90.9±30.4‡	96.5±20.6*

MEDICA 16 indicates β,β'-tetramethylhexadecanedioic acid.
Values are mean±SD for ten 39-week-old rats in each group.
*$P<.05$, †$P<.01$, ‡$P<.001$ vs *cp/cp* control.

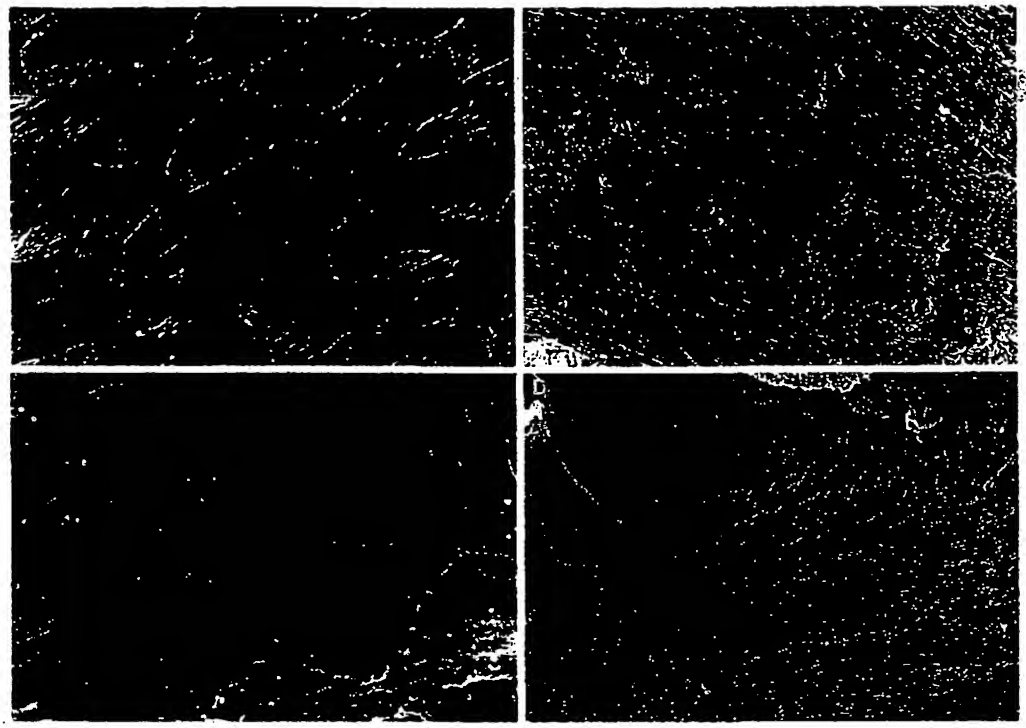

FIG 3. Scanning electron photomicrographs showing raised intimal lesions on the aortic arches of 9-month-old JCR:LA-cp rats. A, +/+ control: score of 0, clean smooth endothelial surface. B, *cp/cp*: score of 1, abnormal endothelial cells overlying a small raised lesion. C, *cp/cp*: score of 2, a moderate raised lesion with highly abnormal overlying endothelial cells. D, *cp/cp*: score of 3, extensive raised lesion with adherent macrophages and limited desquamation of endothelium cells. Bars indicate 10 μm.

pected component of this would be reduced insulin secretion, particularly postprandially, but the present results cannot address this or the metabolic fate of the redirected glucose. These changes in metabolic status occurred without any concomitant toxic effects resulting in histologically detectable abnormalities. At very high doses (650 $mg \cdot kg^{-1} \cdot d^{-1}$), MEDICA 16 has been found to induce hepatic peroxisomal proliferation[27]; however, this effect is species specific and does not occur in humans. No other toxic effects have been found in rodents.

MEDICA 16 is one of the most powerful hypotriglyceridemic agents in the *cp/cp* rat that we have found, causing a 77% decrease in triglycerides at 12 weeks of age[23] and a 71% decrease at 39 weeks of age. The only agent we have studied that is more effective is fluvastatin, which caused a 90% reduction in triglyceride levels in the *cp/cp* rat and resultant levels near those of the +/+ control rats (J.C.R., PhD, unpublished data). We have previously shown that the very marked hypolipidemic effects of MEDICA 16 are due primarily to the inhibition of fatty acid synthesis of the ATP citrate lyase step and resultant decrease in hepatic VLDL secretion.[23]

The overall hypolipidemic, weight-reductive, and antidiabetic effects were reflected by a major decrease in atherosclerosis in MEDICA 16–treated rats. Thus, raised intimal lesions, adherent macrophages, and desquamation of the endothelium were all very significantly reduced. In particular, the lower incidence of areas of adherent macrophages suggests a reduced level of intimal pathophysiological processes in general and of atherogenesis. The improvement in the state of the aortic arch in the MEDICA 16–treated rats is complemented by the lower incidence of stage 4 myocardial lesions. These mature lesions represent a permanent record of the largest of the ischemic lesions cumulated over the life of the rat.[9] Smaller stage 2 lesions, upon fibrosis and contraction, become invisible. In confirmation of this, stage 2 lesions found in the hearts of the MEDICA 16–treated rats were all very small. We have previously reported a similar effect in *cp/cp* male rats treated with nifedipine.[28] In that case, the smaller stage 2 lesions and the reduction in the frequency of stage 4 lesions were suggested to be due to the inhibition of arterial vasospasm secondary to vascular lesions. In the case of MEDICA 16 treatment, we have clear evidence of a reduction in the atherosclerotic damage to the artery. This reduction in myocardial damage should be ascribed to the overall effect exerted by MEDICA 16.

The hypolipidemic effect per se is very dramatic and probably plays an important role, although treatment modes that lower the very high plasma lipid levels of *cp/cp* rats by 50% do not reduce myocardial lesion frequency.[24,29] The results are consistent with our working hypothesis that the insulin-resistant state, through hyperinsulinemia, transient hyperglycemia, or both, is the initiator of the intimal damage. The damage to endothelial cells leads, in the presence of hyperlipidemia, to the development of raised intimal (atherosclerotic) lesions and functional impairment of the vessel wall, with susceptibility to vasospasm.[28,30] MEDICA 16 appears to have sufficient protective effects against both

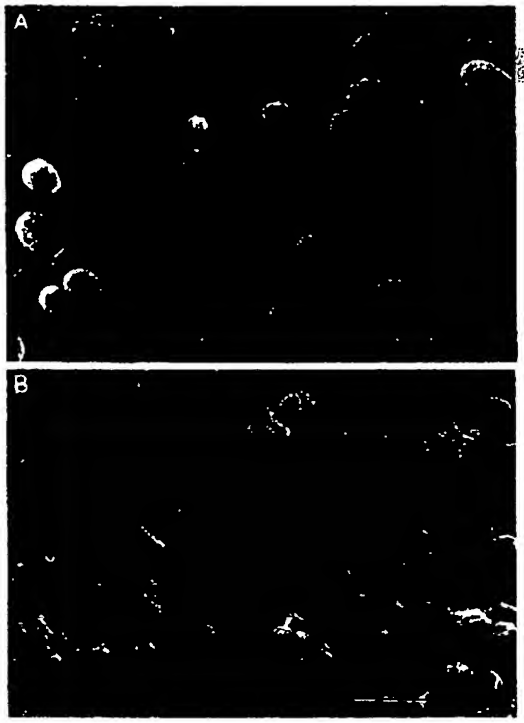

FIG 4. Scanning electron photomicrographs show adherent macrophages on the aortic arches of 9-month-old *cp/cp* male rats. A, score of 2, small focus of adherent macrophages over an area showing endothelial damage. B, score of 3, an area of a large field of macrophages on abnormal endothelium with occasional entrapped erythrocytes. In the absence of macrophages, the score is 0 (no example shown). Bars indicate 10 μm.

abnormalities leading to atherogenesis to markedly inhibit vascular lesion development. The *cp/cp* rat is, in our view, the best available animal model of the metabolic syndrome, showing all the critical elements, including atherosclerotic cardiovascular disease. The evidence to date is consistent with the possibility that the syndrome in the *cp/cp* rat, including the core abnormality of insulin resistance, may originate in abnormal lipid metabolism. The overall beneficial metabolic effects of MEDICA 16 indicate that it affects a central mechanism that links the various parameters of the metabolic syndrome. Although treatment with MEDICA 16 did not completely prevent the development of atherosclerotic lesions in the *cp/cp* rats, the improvement in the status of the aorta was very substantial. Moreover, it was sufficient to cause a major reduction in end-stage disease, ie, ischemic myocardial lesions. This suggests that MEDICA 16 may well offer protective effects against myocardial disease in humans.

TABLE 3. Severity Scores of Aortic Arch Lesions in *cp/cp* Male Rats Treated With β,β′-Tetramethylhexadecanedioic Acid

	Fibrin	Raised Lesions	Macrophages	De-endothelialized Areas
Control	0	2.75±0.71	1.63±1.30	0.63±0.74
MEDICA 16–treated	0.85±1.00	0.95±0.55	0.20±0.26	0.10±0.37
Significance of difference	NS	$P<.01$	$P<.05$	NS

MEDICA 16 indicates β,β′-tetramethylhexadecanedioic acid; NS, not significant.

Values are mean±SD of the severity score, described in "Methods," for ten 39-week-old rats in each group.

TABLE 4. Frequency of Myocardial Lesions in *cp/cp* Male Rats Treated With β,β′-Tetramethylhexadecanedioic Acid

	Lesion Stage			
	1	2	3	4
+/+ control	0	0	0.10±0.32	0.10±0.10
cp/cp control	0.33±0.58	0.33±0.58	0.33±0.58	1.83±1.27
cp/cp MEDICA 16–treated	0.30±0.48	0.48±1.34	0.40±0.52	0.40±0.52*

MEDICA 16 indicates β,β′-tetramethylhexadecanedioic acid.

Values are mean±SD of the frequency of lesions found at each stage for ten 39-week-old rats in each group.

*$P<.01$ vs *cp/cp* control.

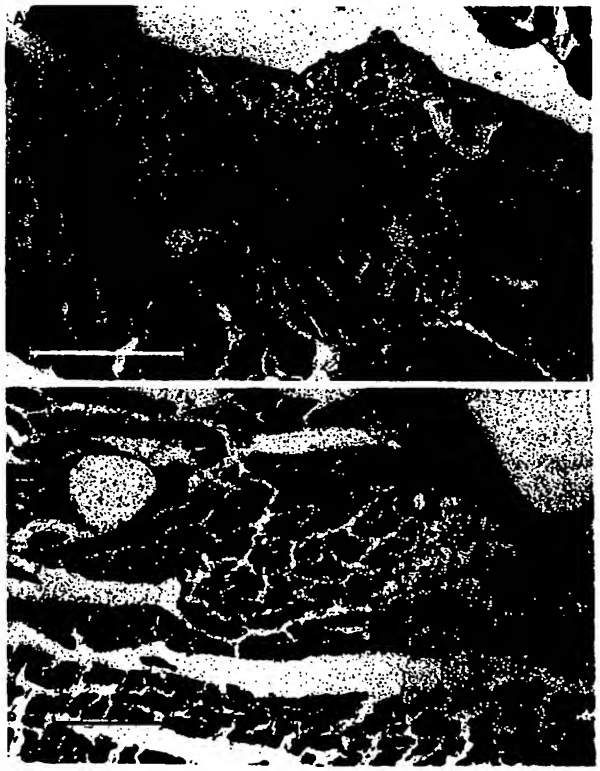

FIG 5. Photomicrographs show representative myocardial lesions from control and β,β′-tetramethylhexadecanedioic acid (MEDICA-16)–treated *cp/cp* male rats. A, typical stage 2 lesion from a control rat. B, very small, typical stage 2 lesion from a MEDICA 16–treated rat. Sections were stained with hematoxylin and eosin. Bars indicate 200 μm.

Acknowledgments

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EXHIBIT C

Applicants: Jacob Bar-Tana
Serial No.: 10/735,439
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Hypolipidemic effect of β,β'-methyl-substituted hexadecanedioic acid (MEDICA 16) in normal and nephrotic rats

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Abstract Treatment of normal or puromycin aminonucleoside-nephrotic rats, kept on a balanced Purina chow diet, with β,β'-tetramethyl-substituted hexadecanedioic acid (MEDICA 16) (Bar-Tana, J., G. Rose-Kahn, and M. Srebnik. 1985. *J. Biol. Chem.* 260: 8404–8410) resulted in an acute reversible inhibition of liver lipogenesis and cholesterogenesis with a concomitant hypolipidemic effect which was sustained as long as the drug was administered. The hypolipidemic effect in normal and nephrotic rats consisted of 70–80% and 40–60% reduction in plasma VLDL-triacylglycerols and cholesterol, respectively, with a respective increase in the HDL-cholesterol/(VLDL + LDL)-cholesterol ratio. The observed hypolipidemic effect was accompanied by a 10-fold decrease in VLDL-apoC-III content with a concomitant enrichment of the VLDL fraction by VLDL remnants having an increased apoB-100/apoB-48 ratio. The pharmacological reduction of VLDL by MEDICA 16 may offer a treatment mode of choice for selected hyperlipidemic states.— **Bar-Tana, J., G. Rose-Kahn, B. Frenkel, Z. Shafer, and M. Fainaru. Hypolipidemic effect of β,β'-methyl-substituted hexadecanedioic acid (MEDICA 16) in normal and nephrotic rats.** *J. Lipid Res.* 1988. 29: 431–441.

Supplementary key words plasma lipids • lipoproteins • dioic acid • hypolipidemic drugs • nephrosis

The capacity of long chain fatty acids and their respective CoA thioesters to act as inhibitors of the lipogenic pathway (1) has initiated the design of inhibitory, nonmetabolic long chain fatty acyl analogues to be exploited as hypolipidemic effectors. β,β'-Methyl-substituted dicarboxylic acids (MEDICA) of C_{14}-C_{18} chain length ($HOOC-CH_2-C(CH_3)_2-(CH_2)n-C(CH_3)_2-CH_2-COOH$) (2) appear to fulfill this role (2, 3), with MEDICA 16 (n = 10) being the most potent of the series. Thus, the ω-carboxyl function interferes with the esterification of the dicarboxylic acid into neutral lipids and phospholipids while still allowing for an ATP-dependent CoA-thioesterification at either carboxylic end (4), and the β,β'-substitution prevents the β-oxidative catabolism of MEDICA compounds by either peroxisomal or mitochondrial systems. As effectors of lipid synthesis, MEDICA compounds were found to potently inhibit liver ATP-citrate lyase in vivo (2) or in cultured rat hepatocytes (3) with a concomitant dose-dependent decrease in liver acetyl-CoA and malonyl-CoA content. Inhibition of the lyase was followed by 80% inhibition of the incorporation of ^3H_2O or acetate into liver esterified fatty acids and 3-β-hydroxysterols under conditions of fat-free carbohydrate-rich feeding (2). Glucose, palmitate, or acetate oxidation as well as the gluconeogenic flux from lactate or the esterification of glycerol into lipids in the presence of added palmitate remained unaffected in vivo or in culture (2, 3). Inhibition of liver lipogenesis and cholesterogenesis by MEDICA compounds was not accompanied by an anorectic or a cathartic effect with reduction in overall net caloric intake (2).

In light of these features of MEDICA compounds it became of interest to evaluate their potential use as hypotriglyceridemic–hypocholesterolemic effectors in the rat in vivo under conditions of a balanced diet which still allows for the production of lipoproteins from exogenous fatty acids and cholesterol. The hypolipidemic effect reported here in the normal as well as the nephrotic rat appears to implicate MEDICA compounds in the catabolism of plasma lipoproteins in addition to their established role in the synthesis of liver lipids.

Abbreviations: PAN, puromycin aminonucleoside; MEDICA, β,β'-methyl-substituted dicarboxylic acid; MEDICA 16, β,β'-methyl-substituted hexadecanedioic acid; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; TLC, thin-layer chromatography; CM, chylomicrons.

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[2]In partial fulfillment of the requirement for a Ph.D. degree of the Hebrew University.

[3]Supported by the Foulkes Foundation.

EXPERIMENTAL PROCEDURES

Animals

Male albino rats of the Hebrew University strain, weighing 150–200 g, were pair-fed nightly meals of ground Purina chow diet (0.1 g/g body weight) consisting of 75–80% of their ad libitum ration and containing 52.5% (w/w) carbohydrates, 18.1% (w/w) proteins, 4.6% (w/w) fat, 5.9% (w/w) cellulose, and 8.5% (w/w) salt-vitamin mixture. Unless otherwise stated, MEDICA 16 was administered by adding the finely powdered drug to the diet for the time periods specified. Dosage was expressed as percent (w/w) of the administered diet. MEDICA 16-treated and nontreated rats consumed the whole food ration under the feeding conditions employed, and the gain in weight following 5 days of treatment amounted to 20.0 ± 3.1 g (n = 12) and 18.2 ± 4.0 (n = 12) for the two respective groups.

Unless otherwise stated, nephrosis was induced by two successive intravenous injections of puromycin aminonucleoside (PAN) (5) at a dose of 7.5 mg/100 g body weight and 5 mg/100 g body weight on the first and third day, respectively.

Plasma triacylglycerol and cholesterol

Total plasma triacylglycerols and cholesterol during follow-up were determined in tail vein blood (0.1–0.2 ml) withdrawn from nonfasted rats at 8–10 AM, using 0.1% NaEDTA (pH 7.0) as an anticoagulant. Cholesterol and triacylglycerols were quantified using the Monotest Cholesterol Enzymatic kit (Boehringer Mannheim, Germany, Cat. No. 237574) and the Biopak Triglyceride Enzymatic kit (Biotrol Paris, France, Cat. No. A 01549), respectively. MEDICA 16 added to the incubation mixture of both enzymatic kits at concentrations of up to 1.25 mM did not interfere with the determination of cholesterol or triacylglycerols.

Lipoprotein analyses

Plasma for lipoprotein analyses was obtained from nonfasted rats at 8–10 AM by vena cava puncture under light ether anaesthesia, using 0.1% NaEDTA as an anticoagulant. All procedures involving lipoprotein isolation and characterization were started immediately after blood drawing and carried out at 4°C.

The distribution of cholesterol and triacylglycerol in plasma lipoproteins was determined by a modification of the LRC protocol (6). The plasma was centrifuged in a Beckman 50.3 Ti rotor for 20 min at 30,000 rpm for chylomicron flotation, followed by centrifuging the recovered chylomicron-deficient plasma for 18 hr at 39,000 rpm for VLDL flotation. The respective chylomicron and VLDL fractions were removed by tube slicing. LDL was precipitated from the 1.006 g/ml infranatant by heparin/$MnCl_2$, and the remaining heparin/$MnCl_2$ supernatant was treated with $NaHCO_3$ (7). Chylomicron cholesterol and triacylglycerol contents were estimated by the difference in the cholesterol and triacylglycerol values between the original plasma and the chylomicron-deficient plasma. VLDL, LDL, and HDL cholesterol and triacylglycerol contents were determined by the respective differences in the cholesterol and triacylglycerol values between the chylomicron-deficient plasma (VLDL + LDL + HDL), the 1.006 g/ml infranatant (LDL + HDL), and the heparin/$MnCl_2$ supernatant (HDL).

The composition of plasma lipoproteins was determined in lipoprotein fractions isolated by sequential density ultracentrifugation as described by Havel, Eder, and Bragdon (8). Chylomicrons, VLDL, LDL, and HDL were isolated by successive flotations for 20 min at 30,000 rpm (chylomicrons), 18 hr at 50,000 rpm at a salt density of 1.006 g/ml (VLDL), 20 hr at 50,000 rpm at a salt density of 1.063 g/ml (LDL), and 44 hr at 50,000 rpm at a salt density of 1.21 g/ml (HDL), respectively. The isolated lipoprotein fractions were refloated at their respective densities and were dialyzed against 400 volumes of 0.15 M NaCl–2 EDTA (pH 7.2). The lipoprotein-protein content of the isolated fractions was determined by the method of Lowry et al. (9) using bovine serum albumin as standard. The lipoprotein-cholesterol and triacylglycerol contents were determined by the respective enzymatic kits. For apoprotein analysis, the respective dialyzed lipoprotein fractions were delipidated with 20 volumes of chloroform–methanol 2:1 followed by 10 volumes of diethylether (10). The protein precipitate was dissolved in 0.1 M sodium phosphate (pH 7.4) containing 1% SDS and 5% 2-mercaptoethanol and heated for 2 min at 100°C. The proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 11% and 4% gels (11). Alternatively, the delipidated lipoproteins were solubilized in 0.015 M Tris-HCl buffer (pH 8.2) containing 6 M urea and subjected to isoelectric focusing (LKB, 2117 Multiphor) between pH 4.0 and 6.0 in 7.5% polyacrylamide gels containing 6 M urea (12). The apoproteins were quantitated by Coomassie blue staining followed by photodensitometry of the stained bands. The differential binding of the stain to apoE, apoC, and apoA-I, and the linearity of dye binding were determined by calibrating the specific dye absorbance of the electrophoresed individual purified apoproteins. The calibration of the dye absorbance of VLDL-apoB was determined by SDS-PAGE of VLDL in 11% gels and the estimation of its apoB content by tetramethylurea precipitation (13). The binding of the stain by VLDL-apoB-100 was assumed to be equal to that of VLDL-apoB-48. The relative binding of the stain amounted to 1.0, 1.25, 3.2, and 10.8 for apoE, apoB, apoC, and apoA-I, respectively, and the binding of the dye was linear within the protein range subjected to SDS-PAGE or isoelectric focusing. The stain binding by apoA-IV was assumed to be the average of that of apoB, E, C, and A-I. Rat VLDL-apoE, VLDL-apoC, and HDL-apoA-I were isolated as previously described (14–16).

VLDL metabolism

Plasma VLDL production was determined in rats injected intravenously under light ether anaesthesia with 0.5 ml of 20% Triton WR-1339 in saline (17). The production of VLDL in the awakened animals was determined by the accumulation of plasma VLDL-triacylglycerol during the period of 40–50 min following the injection of Triton.

The incorporation of ^3H_2O into liver and plasma VLDL lipids was determined by injecting rats intraperitoneally with 10 mCi of ^3H_2O (0.36 Ci/mol) followed 120 min later by sampling the liver and isolating plasma VLDL by sequential density ultracentrifugation as described above. The liver and VLDL lipids were extracted by chloroform–methanol as previously described (2), and the lipid extract was fractionated by TLC as previously described (2, 3). The lipid bands were scraped off the plate and counted in 25% Lumax scintillation fluid in toluene.

Materials

MEDICA 16 was synthesized as previously described (2). Puromycin aminonucleoside and Triton WR-1339 were from Sigma. Ampholyte was from LKB (Sweden). Lumax was from Lumac Application Laboratory (Holland). ^3H_2O was from the Nuclear Research Centre (Negev, Israel). [9,10-^{3}H]Palmitate and [1,2-^{3}H]cholesterol were from Amersham Corp. (U.K.).

RESULTS

Normal rats

Repeated administration of MEDICA 16 to rats fed a Purina balanced diet resulted in a dose-dependent hypotriglyceridemic–hypocholesterolemic effect which reached a 60–80% decrease in plasma triacylglycerols and cholesterol (Fig. 1). The hypolipidemic effect was already established during the first day of treatment (Fig. 1), and was sustained as long as the drug was administered; it was reversed upon withdrawing the drug (not shown).

The plasma lipoprotein profile of rats treated by 0.25% (w/w) MEDICA 16 for 5 days is shown in Table 1. The 65% decrease in total triacylglycerol concentration was accounted for by a respective decrease in the triacylglycerol of chylomicrons and VLDL. The observed decrease in plasma cholesterol consisted of a 75% decrease in VLDL-cholesterol together with a 35% decrease in HDL-cholesterol, while the minor LDL-cholesterol increased threefold. The hypotriglyceridemic–hypocholesterolemic effect was accompanied by a respective decrease in the protein content of plasma VLDL (Table 1). However, since the decrease in the lipid content of VLDL was more pronounced than that of VLDL-protein, a significant increase was observed in the protein/triacylglycerol ratio and the protein/cholesterol ratio of VLDL in MEDICA 16-treated rats.

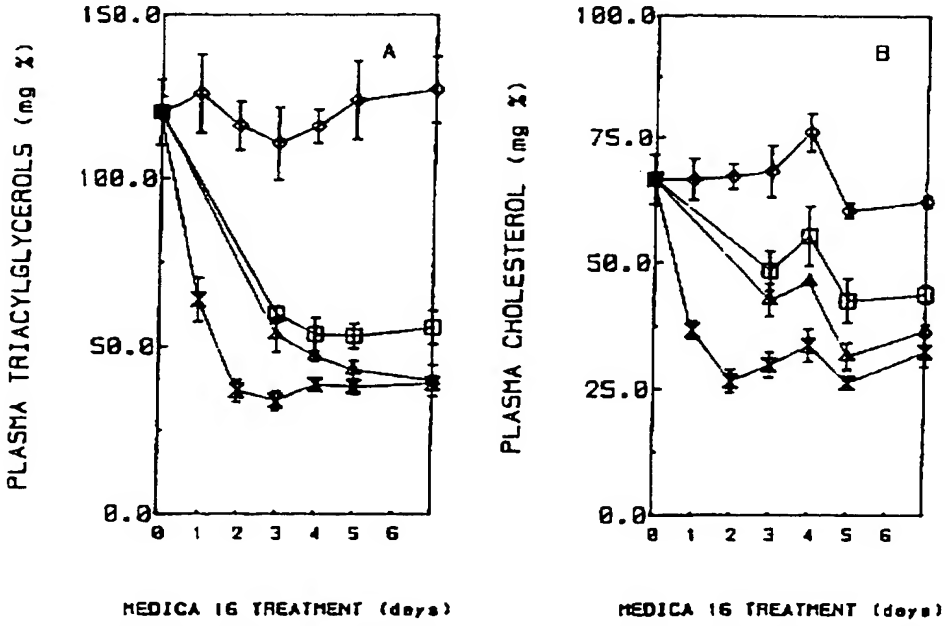

Fig. 1. The hypolipidemic effect of MEDICA 16 in normal rats: time and dose curves. Total plasma triacylglycerol (A) and cholesterol (B) values during follow-up of MEDICA 16-treated and nontreated animals were determined as described in Methods: mean ± SD (n = 5); (◇) 0.0 (nontreated); (□) 0.0625%; (△) 0.125%; and (⧖) 0.25% (w/w) of MEDICA 16 added to the diet.

TABLE 1. Plasma triacylglycerol and cholesterol distribution and the composition of lipoproteins in MEDICA 16-treated normal rats

Composition	Nontreated	MEDICA 16-Treated
Triacylglycerols (mg/dl)		
Total	141.1 ± 30.3	51.3 ± 9.9*
Chylomicrons	62.2 ± 19.2	20.1 ± 6.0*
VLDL	76.7 ± 10.8	25.0 ± 3.9*
LDL	2.1 ± 0.6	2.2 ± 0.3
HDL	3.6 ± 0.3	3.0 ± 0.3
Cholesterol (mg/dl)		
Total	58.0 ± 13.5	32.3 ± 6.6*
VLDL	23.2 ± 5.3	6.1 ± 3.1*
LDL	1.6 ± 0.2	5.1 ± 2.0*
HDL	30.4 ± 4.9	20.6 ± 4.2*
Protein (mg/dl)		
VLDL	13.27 ± 3.32	6.60 ± 1.03*
HDL	73.14 ± 11.78	54.78 ± 11.17*
Composition ratios		
VLDL-protein/VLDL-triacylglycerols	0.173 ± 0.021	0.264 ± 0.096*
VLDL-protein/VLDL-cholesterol	0.570 ± 0.070	1.080 ± 0.110*
VLDL-cholesterol/VLDL-triacylglycerols	0.169 ± 0.025	0.224 ± 0.030*
HDL-protein/HDL-cholesterol	2.406 ± 0.225	2.659 ± 0.154
HDL-triacylglycerols/HDL-cholesterol	0.209 ± 0.086	0.298 ± 0.054

Male rats fed a Purina chow diet were treated with 0.25% (w/w) MEDICA 16 added to the diet for 3 days. The distribution of cholesterol and triacylglycerol in plasma lipoproteins was determined by the modified LRC protocol as described in Methods. The lipoprotein composition was determined in the washed-dialyzed lipoprotein fractions isolated by sequential density ultracentrifugation as described in Methods. The protein content was calculated from the respective composition ratios and the lipid content of the respective particles; mean ± SD (n = 5).
*Significantly different from the respective nontreated value; $P < 0.05$.

The apoprotein composition in MEDICA 16-treated rats is shown in Table 2. The most significant changes were observed in the compositions of VLDL (Fig. 2A, B, C) and HDL (Fig. 2D, E) while that of LDL remained unaffected. Thus, the VLDL-apoB-100/VLDL-apoB-48 ratio amounted to 1.00 and 0.46 in MEDICA 16-treated and nontreated rats, respectively, thus pointing to a relative enrichment in apoB-100 as a result of MEDICA 16 treatment (Fig. 2B). Furthermore, the fractional content of VLDL-apoC decreased by 7.5-fold with a concomitant 9-fold increase in the apoE/apoC ratio of VLDL in MEDICA 16-treated rats. The specificity of the MEDICA 16 effect with respect to VLDL-apoC could be further realized by analyzing the plasma concentration of each of the VLDL-apoproteins as calculated by multiplying the fractional abundance of the respective VLDL-apoproteins (Table 2) by the total VLDL-protein of treated and nontreated rats (Table 1). Thus, the plasma concentrations of (apoB-100 + apoB-48), apoE, and apoC in VLDL amounted to 3.5, 5.8, and 4.0 mg/dl of plasma in nontreated rats as compared to 2.7, 3.6, and 0.3 mg/dl of plasma in MEDICA 16-treated rats. The observed decrease in VLDL-apoC was further analyzed by isoelectric focusing of the VLDL apoproteins (Fig. 2C). The relative abundance in terms of densitometric units amounted to 52, 116, 35, and 110 as compared to 23, 40, 8, and 66 for apoC-II, apoC-III-0, apoC-III-1, 2, and apoC-III-3 of nontreated and MEDICA 16-treated VLDL, respectively. Thus, in the light of the relative abundance of apoC-III, most of the overall decrease induced by MEDICA 16 treatment in VLDL-apoC was accounted for by the respective decrease in apoC-III. However, no preferential enrichment was observed in VLDL-apoC-II.

The apoprotein composition of the HDL fraction in MEDICA 16-treated rats was characterized by a twofold decrease in the fractional abundance of HDL-apoC and a twofold increase in the fractional abundance of HDL-apoE with a concomitant fourfold increase in the apoE/apoC ratio. The plasma concentrations of HDL-apoA-I, HDL-apoE, and HDL-apoC in nontreated rats were 23.2, 11.0, and 25.6 mg/dl of plasma, respectively, as compared to 16.4, 18.2, and 9.5 mg/dl, respectively, in MEDICA 16-treated rats. The relative abundance of the HDL-apoC subfractions in terms of densitometric units were 90, 126, 13, and 68 as compared to 62, 51, 2, and 51 for apoC-II, apoC-III-0, apoC-

TABLE 2. Apolipoprotein composition of MEDICA 16-treated normal rats

Composition	% Composition	
	Nontreated	MEDICA 16-Treated
VLDL		
ApoB-100	8.3	20.5
ApoB-48	18.1	20.5
ApoE	43.4	54.9
ApoC	30.2	4.1
ApoB-100/apoB-48	0.46	1.0
ApoE/apoC	1.44	13.4
LDL		
ApoB-100	63.4	66.7
ApoB-48	9.5	6.8
ApoE	27.2	26.6
HDL		
ApoE	15.1	33.2
ApoC	35.0	17.4
ApoA-I	31.7	29.9
ApoA-IV	18.1	19.5
ApoE/apoC	0.43	1.9

Male rats fed a Purina chow diet were treated with 0.25% (w/w) MEDICA 16 added to the diet for 5 days. The individual lipoprotein fractions of pooled plasma of five to ten treated and nontreated rats were isolated by sequential density ultracentrifugation as described in Methods and the apoprotein composition was determined by SDS-PAGE as described in Methods. The photodensitometric values were corrected for the differential binding of the stain as described in Methods; mean of four experiments. (The % composition presented did not vary by more than 20% of the respective individual composition values.)

III-1, 2, and apoC-III-3 of nontreated and MEDICA 16-treated rats, respectively (Fig. 2E), thus resulting in a 1.4-fold increase in the HDL-apoC-II/HDL-apoC-III ratio.

Nephrotic rats

Nephrosis may offer a hyperlipidemic model system for studying the potential of hypolipidemic agents. Indeed, PAN-induced nephrosis in rats (Fig. 3) resulted in 10- to 20-fold progressive increase in plasma triacylglycerol and cholesterol levels as previously reported (18–20), which was sustained at least for 14 days without reverting spontaneously to the normal level. PAN-induced nephrosis was accompanied by the appearance of a major LDL fraction which contributed about 35% to plasma cholesterol, thus making possible the evaluation of MEDICA effect on LDL-cholesterol in the rat. The induction of PAN-nephrosis in MEDICA 16-treated rats resulted in a restrained hyperlipidemic state compared to that observed in nontreated rats (Fig. 3). The effect exerted by MEDICA 16 could not be ascribed to interference of the drug with the induction of PAN-nephrosis, since MEDICA treatment was found to reverse the lipid pattern in an already established PAN-nephrotic state. Thus, treatment of PAN-nephrotic rats by MEDICA 16 for 5 days, starting on the 9th day following the induction of nephrosis by PAN, resulted in an almost complete reversion of plasma triacylglycerol back to the normal level, with a concomitant 50% decrease in plasma cholesterol (Fig. 4).

The plasma lipoprotein profile accounting for the hypolipidemic effect in MEDICA 16-treated nephrotic rats is shown in Table 3, and may be compared to that observed in normal rats (Tables 1 and 2). The hypotriglyceridemic effect induced by MEDICA 16 could be ascribed to an 80% decrease in the triacylglycerol content of chylomicrons and VLDL, while the overall hypocholesterolemic effect resulted from 65%, 70%, and 50% decreases in chylomicron-, VLDL-, and LDL-cholesterol, respectively, with no change in HDL-cholesterol. The relatively high fractional abundance of HDL-cholesterol in the nephrotic rat (Table 3) (18) taken together with the lack of MEDICA 16 effect on HDL-cholesterol accounts for the limited overall decrease in total plasma cholesterol in MEDICA 16-treated nephrotic rats (Fig. 4), with a concomitant increase in the HDL-cholesterol/(VLDL + LDL)-cholesterol ratio from 0.43 to 1.09.

The abundance of VLDL-apoB, VLDL-apoE, and VLDL-apoC in PAN-nephrotic rats amounted to 46.8, 56.8, and 35.5 mg/dl of plasma, respectively, in nontreated rats as compared to 12.4, 17.1, and 6.5, respectively, in MEDICA 16-treated rats (Table 2 and Table 4). Thus, the specific decrease in VLDL-apoC induced by MEDICA 16 treatment appears to be somewhat masked in the nephrotic rat by the overall decrease in the content of VLDL proteins. Also, similar to the increase in the fractional abundance of VLDL-apoB-100 induced by MEDICA 16 in normal rats, the apoB-100/apoB-48 ratio in nephrotic rats amounted to 0.88 and 1.9 in nontreated and MEDICA 16-treated nephrotic rats, respectively. It is worth noting that the increase in the fractional abundance of apoB-100 due to nephrosis was further accentuated by MEDICA 16 treatment, and the combined effect of nephrosis and MEDICA 16 treatment resulted in apoB-100 becoming the major apoB conponent of VLDL in MEDICA 16-treated nephrotic rats. The HDL-apoprotein profile observed in nephrotic rats was characterized by a significant decrease in the fractional abundance of apoE and apoA-IV as previously described (18, 19). Treatment of nephrotic rats with MEDICA 16 resulted in an increase in HDL-apoE at the expense of HDL-apoC, while apoA-I remained essentially unaffected.

VLDL metabolism in MEDICA 16-treated rats

The hypolipidemic effect of MEDICA 16 with respect to VLDL was further pursued by studying the synthesis and secretion of liver VLDL in normal and nephrotic MEDICA 16-treated rats. As shown in Table 5, ^3H_2O incorporation into total liver lipids and plasma VLDL lipids was potently inhibited by MEDICA 16 in PAN-nephrotic

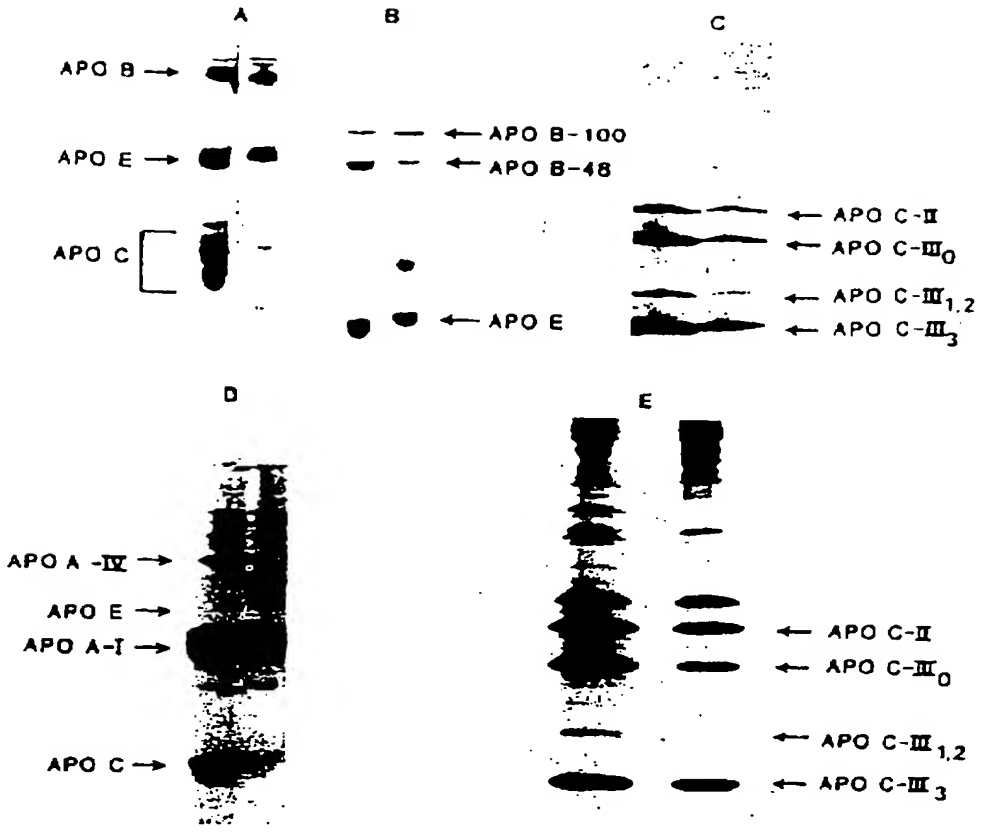

Fig. 2. The apoprotein composition of VLDL and HDL in MEDICA 16-treated rats. Rats were treated with 0.25% (w/w) MEDICA 16 for 5 days. Pooled plasma VLDL and HDL from five to ten nontreated (left lane) or treated (right lane) rats were isolated, delipidated, and analyzed by SDS-PAGE or isoelectric focusing as described in Methods. A, SDS-PAGE of VLDL-apoproteins in 11% gels (100 μg); B, SDS-PAGE of VLDL-apoproteins in 4% gels (100 μg); C, Isoelectric focusing of VLDL-apoproteins (200 μg); D, SDS-PAGE of HDL-apoproteins in 11% gels (100 μg); E, Isoelectric focusing of HDL-apoproteins (150 μg).

rats, thus reflecting the established capacity of MEDICA compounds to inhibit VLDL production (2, 3) under conditions of nephrosis where de novo lipogenesis and cholesterogenesis constitute a major flux in liver lipids synthesis (21). It it worth noting that inhibition of ^3H_2O incorporation into plasma VLDL lipids was actually much more pronounced than the reduction in the mass of the lipid constituents of plasma VLDL (Table 5 vs. Table 3); the difference presumably reflects the specific inhibition of liver lipogenesis by MEDICA 16 while the esterification flux of endogenous fatty acids remains unaffected (2, 3).

The hypolipidemic effect observed in normal rats kept on a carbohydrate-rich, fat-free diet, where de novo lipogenesis and cholesterolgenesis constituted a major route for liver VLDL production, was similarly accounted for by 65% inhibition in liver VLDL production (Table 6) with a concomitant inhibition in ^3H_2O incorporation triacylglycerol and cholesterol (2). However, the demic effect of MEDICA 16 with respect to plasm could not be exclusively ascribed to inhibition c VLDL production since it could still be verified u: ditions of starvation, where VLDL production wa repressed and could not be further inhibited by M 16 treatment (Table 6).

DISCUSSION

Treatment of normal rats fed a balanced Puri diet with MEDICA 16 resulted in an acute and r hypolipidemic effect that was sustained as long as was administered. The hypolipidemic effect amo

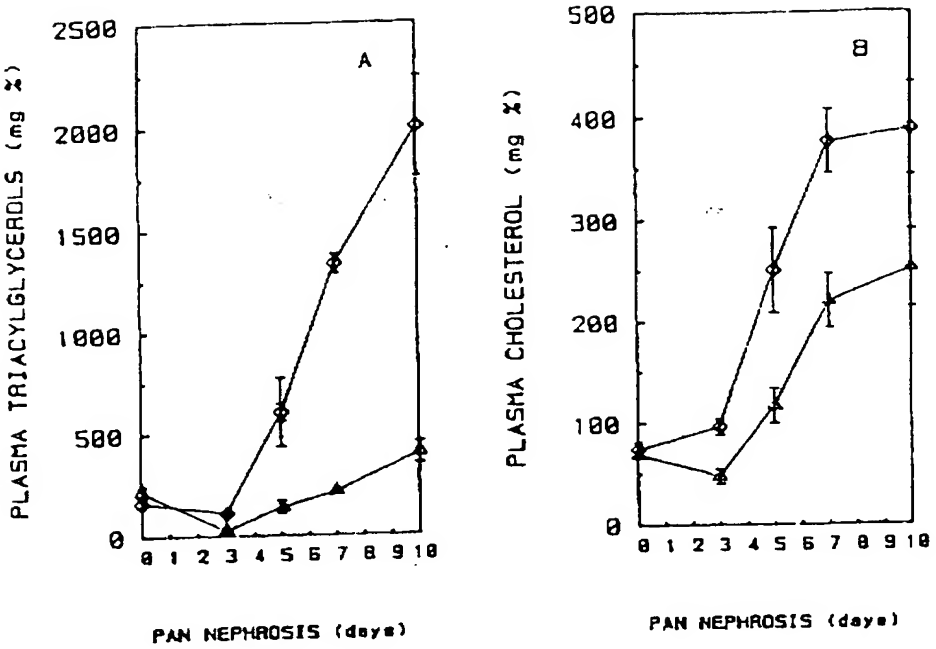

Fig. 3. PAN-nephrosis in MEDICA 16-treated rats. Nontreated rats (○) or rats treated with 0.25% (w/w) MEDICA 16 added to the diet from the 1st day (△) were injected intravenously once with 10 mg of PAN/100 g body weight on the 1st day. Plasma total triacylglycerol (A) and cholesterol (B) were determined on the specified days following PAN administration as described in Methods; mean ± SD (n = 5).

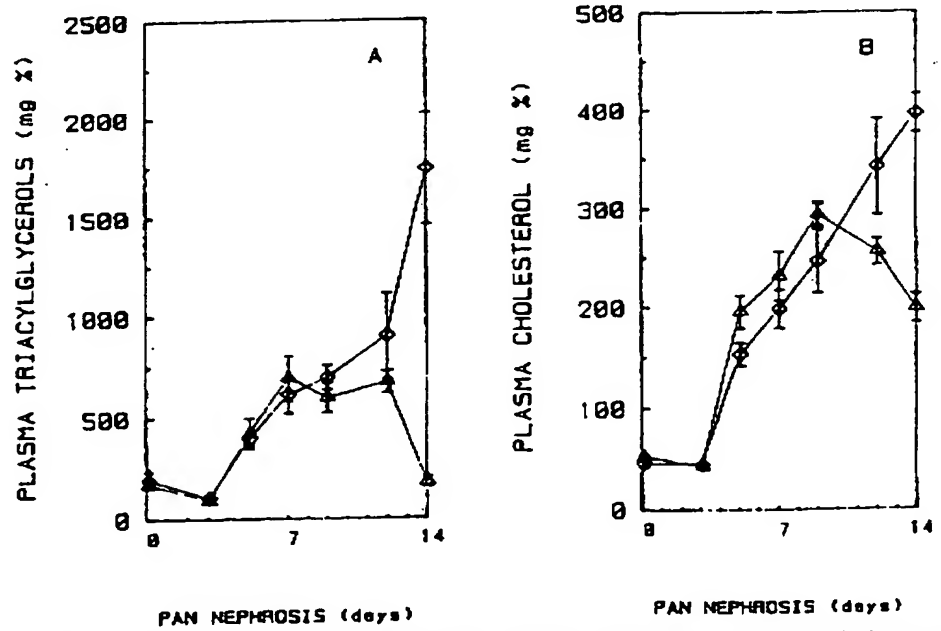

Fig. 4. The hypolipidemic effect of MEDICA 16 in PAN-nephrosis. PAN nephrosis was induced by intravenous injections of PAN on the 1st day and the 3rd days as described in Methods. MEDICA 16-treated rats (△) were dosed with 0.25% (w/w) MEDICA 16 added to the diet from the 9th day on. Nontreated rats (○) were fed the normal diet. Plasma total triacylglycerol (A) and cholesterol (B) were determined on the specified days as described in Methods; mean ± SD (n = 4).

TABLE 3. Plasma triacylglycerol and cholesterol distribution and the composition of lipoproteins in MEDICA 16-treated PAN-nephrotic rats

Composition	Nontreated	MEDICA 16-Treated
Triacylglycerols (mg/dl)		
Total		
Chylomicrons	1128.7 ± 529.9 (9)	254.1 ± 122.9 (9)*
VLDL	500.7 ± 233 ± (9)	106.8 ± 55.3 (9)*
LDL	801.3 ± 393.1 (9)	170.3 ± 79.1 (9)*
HDL	24.8 ± 10.2 (9)	9.1 ± 4.5 (9)*
	11.0 ± 0.6 (9)	8.9 ± 1.4 (9)
Cholesterol (mg/dl)		
Total		
Chylomicrons	414.7 ± 101.5 (9)	246.3 ± 42.8 (9)*
VLDL	48.5 ± 13.6 (9)	15.9 ± 13.0 (9)*
LDL	124.4 ± 55.9 (9)	36.8 ± 19.9 (9)*
HDL	136.4 ± 61.8 (9)	78.3 ± 39.6 (9)*
	121.8 ± 17.9 (9)	125.1 ± 17.7 (9)
Protein (mg/dl)		
Chylomicrons		
VLDL	56.08 ± 26.14 (9)	42.61 ± 22.07 (9)
LDL	141.03 ± 69.19 (9)	36.04 ± 14.79 (9)*
HDL	97.44 ± 38.50 (9)	37.43 ± 4.36 (9)*
	231.30 ± 34.00 (9)	222.43 ± 31.47 (9)
Composition ratios		
CM-protein/CM-triacylglycerols	0.112 ± 0.044 (7)	0.399 ± 0.124 (7)*
CM-cholesterol/CM-triacylglycerols	0.065 ± 0.033 (7)	0.064 ± 0.024 (7)
VLDL-protein/VLDL-triacylglycerols	0.176 ± 0.133 (5)	0.211 ± 0.103 (5)
VLDL-cholesterol/VLDL-triacylglycerols	0.093 ± 0.030 (5)	0.085 ± 0.028 (5)
LDL-protein/LDL-cholesterol	0.623 ± 0.076 (9)	0.478 ± 0.110 (9)
LDL-triacylglycerols/LDL-cholesterol	0.242 ± 0.096 (5)	0.180 ± 0.067 (5)
HDL-protein/HDL-cholesterol	1.899 ± 0.191 (9)	1.778 ± 0.134 (9)
HDL-triacylglycerols/HDL-cholesterol	0.117 ± 0.057 (5)	0.074 ± 0.012 (5)

PAN-nephrotic rats fed a Purina chow diet were treated with 0.25% (*w/w*) MEDICA 16 for 5 days starting on the 9th day as described in Fig. 4. The distribution of cholesterol and triacylglycerol in plasma lipoproteins was determined by the modified LRC protocol as described in Methods. The lipoprotein composition was determined in the washed-dialyzed lipoprotein fractions isolated by sequential density ultracentrifugation as described in Methods. The protein content was calculated from the respective composition ratios and the lipid content of the respective particle; Mean ± SD (number of animals).

*Significantly different from the respective nontreated value; $P < 0.05$.

70–80% and 40–60% reduction in plasma triacylglycerol and cholesterol, respectively, and was due essentially to a decrease in the lipid content of chylomicrons and VLDL. The decrease in the triacylglycerol and cholesterol content of the triacylglycerol-rich lipoproteins was accompanied by specific significant changes in the composition of VLDL which consisted of a tenfold decrease in its apoC content and a twofold increase in its apoB-100/apoB-48 ratio. The decrease in VLDL-apoC reflected an overall decrease in plasma apoC rather than a selective transfer of apoC from VLDL to HDL. In the light of the fractional abundance of VLDL-apoC-III, most of the decrease in VLDL-apoC could be accounted for by that of apoC-III. The extent of the hypolipidemic effect of MEDICA 16 in PAN-nephrotic rats reflects the lipid-lowering potential of MEDICA compounds in pathological hyperlipidemic states.

The hypolipidemic effect of MEDICA 16 is qualitatively similar to that observed in starvation with respect to the fractional enrichment in VLDL-apoE and VLDL-apoB-100 (22–24), and the concomitant decrease in liver apoC secretion (25). However, the MEDICA effect could not be ascribed to reduction in the overall net caloric intake as a result of either anorectic or cathartic effects of the drug. Thus, the food comsumption of MEDICA 16-treated rats kept under ad libitum feeding conditions was similar to that of nontreated, age-matched rats, whether normal or nephrotic. Moreover, the hypolipidemic effect was observed here under pair-feeding conditions, where both nontreated and MEDICA 16-treated rats were offered only 75–80% of their ad libitum daily ration and the offered ration was fully consumed by both experimental groups. Also, the daily amount of stool excretion was similar in treated and nontreated rats. Hence, the hypolipidemic effect of MEDICA 16 reflects a metabolic modality rather than being due to a decrease in overall net caloric intake.

The hypolipidemic effect of MEDICA compounds with respect to plasma VLDL was previously observed in rats fed a carbohydrate-rich fat-free diet (2) and was ascribed there

TABLE 4. Apolipoprotein composition of MEDICA 16-treated PAN-nephrotic rats

Composition	% Composition	
	Nontreated	MEDICA 16-Treated
VLDL		
ApoB-100	15.6	22.6
ApoB-48	17.6	11.9
ApoE	41.6	47.5
ApoC	25.2	18.1
ApoB-100/apoB-48	0.88	1.90
ApoE/apoC	1.65	2.62
LDL		
ApoB-100	44.0	56.2
ApoB-48	5.4	9.2
ApoE	50.6	34.6
HDL		
ApoE	4.1	15.7
ApoC	43.5	32.4
ApoA-I	47.8	50.8
ApoA-IV	4.4	1.1
ApoE/apoC	0.09	0.48

PAN-nephrotic rats fed a Purina chow diet were treated with 0.25% (w/w) of MEDICA-16 in the diet for 5 days as described in Fig. 4. The individual lipoprotein fractions of pooled plasma of five to eight treated and nontreated rats were isolated by sequential density ultracentrifugation as described in Methods, and their respective apoprotein composition was determined by SDS-PAGE followed by photodensitometry of the Coomassie blue-stained bands; mean of two experiments. (The % composition presented did not vary by more than 10% of the respective composition values of the two experiments.)

to MEDICA inhibition of the lipogenic and cholesterogenic fluxes by MEDICA compounds acting as reversible, citrate-competitive inhibitors of liver ATP-citrate lyase as well as irreversible inhibitors of the cholesterogenic pathway at a site beyond the HMG-CoA reductase (2, 3). The inhibition of lipogenesis and cholesterogenesis by MEDICA 16 was confirmed here in nephrotic rats (Table 5), and may indeed account for the hypolipidemic effect of MEDICA compounds under conditions of carbohydrate-rich, fat-free diet or nephrosis where endogenous lipogenesis and cholesterogenesis constitute a major flux of liver lipid synthesis. The present results indicate, however, that the hypolipidemic effect of MEDICA 16 may still be expressed under conditions where VLDL synthesis is already repressed by starvation and cannot be further inhibited by MEDICA 16 (Table 6), thus implicating MEDICA compounds in VLDL catabolism apart from their established involvement in VLDL synthesis. Moreover, the increase in VLDL-apoB-100/apoB-48 ratio and the decrease in VLDL-apoC content in MEDICA 16-treated rats further point to the relative enrichment of the VLDL fraction by VLDL remnants (26, 27). Also, the consistent increase in the LDL fraction which accompanies the decrease in VLDL in MEDICA 16-treated normal rats, as well as the restrained decrease in LDL in MEDICA 16-treated nephrotic rats under conditions of a pronounced decrease in their VLDL, seem to corroborate the presumed role of MEDICA 16 in VLDL catabolism. Furthermore, the hypochylomicronemic effect of MEDICA 16 (Tables 1 and 3) points to the hypolipidemic capacity of this drug under conditions where lipogenesis and cholesterogenesis are replaced altogether by exogenous dietary lipids. Since MEDICA 16 does not affect chylomicron synthesis, assembly, and secretion into mesenteric lymph (Frenkel, B., et al., unpublished results), the observed hypochylomicronemic effect could not be accounted for by MEDICA inhibition of chylomicron production. Preliminary results have indeed indicated that the fractional clearance rates of palmitate- or cholesteryl ester-labeled VLDL and chylomicron particles prepared in normal rats and injected into MEDICA 16-treated rats were increased 6- to 10-fold. Hence, the hypolipidemic effect of MEDICA

TABLE 5. The incorporation of ^3H_2O into liver and plasma VLDL in MEDICA 16-treated and nontreated PAN-nephrotic rats

Fraction	^3H_2O Incorporation into Liver Lipids (μmol/g liver per 120 min)		^3H_2O Incorporation into Plasma VLDL (μmol/ml plasma per 120 min)	
	Nontreated	MEDICA 16-Treated	Nontreated	MEDICA 16-Treated
Total lipids	76.9 ± 22.8	21.8 ± 4.1*	1.63 ± 0.15	0.12 ± 0.01*
Triacylglycerols	30.5 ± 18.0	4.7 ± 2.8*	0.44 ± 0.05	0.03 ± 0.00*
Phospholipids	21.4 ± 10.4	6.0 ± 2.3*	0.11 ± 0.01	0.01 ± 0.01*
FFA	15.4 ± 6.2	4.0 ± 2.1*	0.84 ± 0.41	0.08 ± 0.06*
Cholesterol	2.5 ± 0.0	1.7 ± 0.6*	0.03 ± 0.01	n.d.
Cholesteryl ester	1.9 ± 0.2	0.7 ± 0.1*	0.01 ± 0.01	n.d.

PAN nephrosis was induced by two intravenous injections of PAN on the first and third days as described in Methods. MEDICA 16 (0.25%, w/w) was added to the diet from the 9th day on. Nontreated rats were fed the vehicle diet. On the 14th day the rats were injected with ^3H_2O and the incorporation of radioactivity into liver lipids and plasma VLDL was determined as described in Methods. The liver weight amounted to 11.0 ± 0.9 g (5.5% of body weight) and 11.8 ± 1.2 g (5.9% of body weight) in nontreated and MEDICA 16-treated rats, respectively; mean ± SD (n = 4); n.d., not detectable.

*Significantly different from the respective nontreated value, $P < 0.05$.

TABLE 6. Plasma VLDL production in MEDICA 16-treated rats

Starvation Period	Basal Plasma Triacylglycerols (mg/dl)		Plasma VLDL Production (mg triacylglycerol/min per dl of plasma)	
	Nontreated	MEDICA 16-Treated	Nontreated	MEDICA 16-Treated
	160 ± 40	49 ± 7	9.0 ± 1.1	3.1 ± 0.5
24 hr	80 ± 24	42 ± 13	2.4 ± 0.7	3.0 ± 0.6
48 hr	66 ± 20	44 ± 12	2.7 ± 0.7	2.9 ± 0.6

Male rats weighing 150 g were fed a carbohydrate-rich, fat-free diet ad libitum followed by starvation for the indicated time. MEDICA 16 was added to the powered diet at a concentration of 0.25% (w/w) and was continued during the starvation period (45 mg MEDICA 16/day) in 1% methylcellulose administered by an intragastric tube. Basal plasma triacylglycerols were determined in tail blood samples of ether-anaesthesized rats. Plasma VLDL production was determined by the difference in plasma triacylglycerol 40–50 min following the injection of Triton WR-1339 as described in Methods; mean ± SD (n = 4).

16 with respect to plasma VLDL in rats kept on a balanced Purina diet may be ascribed to both inhibition of liver VLDL production and activation of plasma VLDL catabolism. The activation of catabolism of the triacylglycerol-rich particles could be of major relevance either during the transition from the normolipidemic steady state of the nontreated animal to the hypolipidemic steady state of the MEDICA 16-treated animal, in the course of the decrease of VLDL under physiological conditions where lipogenesis and cholesterogenesis are already repressed (e.g., starvation), or in the course of the hypochylomicronemic effect induced by MEDICA 16 treatment. The possible role of the decreased plasma apoC-III in the activation of plasma VLDL and chylomicron catabolism (28, 29) in MEDICA 16-treated rats is now under investigation.

The overall pattern induced by MEDICA 16 treatment in rats is remarkably similar in many respects to that recently described in human subjects with a genetic deficiency in apoC-III and apoA-I (30, 31). In both cases the hypolipidemic effect with respect to VLDL was extensive and accompanied by apoC-III deficiency with a concomitant significant increase in the fractional clearance rate of normal VLDL particles injected into apoC-III-deficient subjects. The HDL fraction was, however, preserved upon MEDICA 16 treatment, while it was essentially absent under conditions of a genetic deficiency in apoA-I, thus resulting in premature atherosclerosis. The pharmacological reduction of VLDL-apoC-III by MEDICA 16 may thus help in dissecting the contribution made by apoC-III to the overall metabolism of lipoproteins under conditions where apoA-I is conserved.

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EXHIBIT D

Applicants: Jacob Bar-Tana
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Exhibit D 4.1.(3)

Sensitization to Insulin Induced by β,β′-Methyl–Substituted Hexadecanedioic Acid (MEDICA 16) in Obese Zucker Rats In Vivo

Nina Mayorek, Bella Kalderon, Etty Itach, and Jacob Bar-Tana

β,β′-methyl–substituted hexadecanedioic acid (MEDICA 16) consists of a nonmetabolizable long-chain fatty acid designed to probe the effect exerted by fatty acids on insulin sensitivity. The effect of MEDICA 16 was evaluated in insulin-resistant Zucker (*fa/fa*) rats in terms of liver, muscle, and adipose tissue response to clamped euglycemic hyperinsulinemia in vivo. Nontreated Zucker rats were insulin resistant, maintaining basal rates of total-body glucose disposal, glucose production in liver, free fatty acid (FFA) flux into plasma, and FFA reesterification in adipose tissue, irrespective of the insulin levels induced. MEDICA 16 treatment resulted in an insulin-induced decrease in hepatic glucose production, together with an insulin-induced increase in total-body glucose disposal. Intracellular reesterification of lipolysed FFA in adipose tissue was specifically activated by MEDICA 16, resulting in a pronounced decrease in FFA release, with a concomitant decrease in plasma FFA. In conclusion, MEDICA 16 treatment results in the sensitization of liver, muscle, and adipose tissue to insulin in an animal model for obesity-induced insulin resistance. *Diabetes* 46:1958–1964, 1997

Long-chain fatty acids have been repeatedly reported to modulate carbohydrate, lipid, and protein metabolism, as well as the onset and progression of the metabolic syndrome driven by insulin resistance (1). This modulatory effect is usually ascribed to fatty acid oxidation at the expense of glucose utilization (2,3) or to effects exerted by fatty acids serving as precursors for adipose triglycerides (4) and membrane phospholipids (5). The effect exerted by downstream metabolic products of fatty acids could, however, mask direct modulatory effects exerted by the fatty acid precursor or its immediate metabolite (e.g., CoA-thioester). This putative modulatory capacity of fatty acids has initiated the synthesis of nonmetabolizable analogs of long-chain fatty acids for probing the role played by long-chain fatty acids in the metabolic syndrome. Substituted dicarboxylic acids may fulfill this objective, with β,β′-methyl–substituted hexadecanedioic acid (MEDICA 16) being the most studied homolog of the series (6).

MEDICA 16 treatment results in hypolipidemia, calorigenesis, and amelioration of NIDDM in respective animal models. The hypolipidemic effect consists of a pronounced decrease in the triacylglycerol and cholesterol content of plasma chylomicrons and VLDL in both normolipemic (7) as well as hyperlipemic animal models (7,8). The hypolipidemic effect is accounted for by the activation of clearance of plasma chylomicrons and VLDL as a result of a decrease in plasma apolipoprotein C-III leading to activation of lipoprotein lipase and deinhibition of triglyceride-rich lipoprotein uptake by respective liver receptors (9,10). The decrease in plasma apolipoprotein C-III is due to displacement of the activatory transcription hepatocyte nuclear factor (HNF)-4α from its cognate C3P enhancer of the *apo* C-III gene promoter by MEDICA 16–activated PPARα/RXR resulting in transcriptional suppression of the liver *apo* C-III gene by MEDICA 16 (11).

The hypolipidemic effect of MEDICA 16 is accompanied by MEDICA 16–induced calorigenesis, similar in nature to that induced by thyroid hormones. The calorigenic-thyromimetic activity is liver specific and characterized by a decrease in liver phosphate potential and liver redox potential with a concomitant increase in oxygen consumption (12,13). This activity is due to *1*) direct mitochondrial action of the drug, resulting in decoupling of mitochondrial oxidative phosphorylation (O. Hermesh, B.K., J.B.-T., unpublished observations), *2*) direct inhibition of ATP-citrate lyase with concomitant decrease in malonyl-CoA content (14), and *3*) transcriptional activation of liver genes classically considered to be thyroid hormone dependent (e.g., malic enzyme, mitochondrial S14, glycerol-3-phosphate dehydrogenase) (15). Transcriptional activation by MEDICA 16 in the context of the malic enzyme gene was accounted for by MEDICA 16–induced binding of the PPARα/RXRα heterodimer to a distinct 5′-flanking PPRE enhancer of the malic enzyme gene promoter (16).

The antidiabetic effect of MEDICA 16 was verified in several animal models for obesity-induced diabetes characterized by hyperinsulinemia with normoglycemia or hyperglycemia (17–19). The tolerance to glucose was essentially normalized, and plasma insulin levels were found to be pronouncedly decreased by MEDICA 16 treatment, approaching those of caloric-restricted animals or those observed in lean albino rats. In contrast to animal models for obesity-induced diabetes, streptozotocin-induced diabetes could not be improved by MEDICA 16 treatment, thus further indicating that the improved glucose handling in MEDICA 16–treated animals requires insulin and could reflect sensitization to

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FFA, free fatty acid; GC-MS, gas chromatography–mass spectrometer; HGP, hepatic glucose production; MEDICA 16, β,β′-methyl–substituted hexadecanedioic acid.

insulin. This putative insulin-sensitizing effect of MEDICA 16 was evaluated here in vivo in insulin-resistant Zucker (*fa/fa*) rats in terms of the liver, muscle, and adipose tissue response to clamped euglycemic hyperinsulinemia. A potent insulin-sensitizing activity of MEDICA 16 could be exemplified in all three tissues.

RESEARCH DESIGN AND METHODS

Animals. Zucker (*fa/fa*) obese rats aged 8–9 weeks were individually housed in metabolic cages with free access to water and standard rat diet (55% carbohydrate, 20% protein, 4.5% fat, 10% moisture, 3.4% cellulose, 5% ash). Weight gain and food intake were recorded daily. After 1 week of adaptation, MEDICA 16 was dosed in the diet by stepwise addition of the drug during a period of 2 weeks, reaching a final dose of 390 mg · kg^{-1} body wt · day^{-1}. The final dose was maintained for a period of 4–8 weeks. The steady-state plasma levels attained in nonfasting animals amounted to 247 ± 32 µg MEDICA 16/ml. Postabsorption plasma levels amounted to 32 ± 2.7 µg MEDICA 16/ml.

Clamp studies. The 15-h fasted animals weighing 500–600 g were placed in a restriction cage and were cannulated under local anesthesia with lignocaine (20) through the tail artery and vein for blood sampling and for priming and constant infusion, respectively. After catheter placement, animals were released to their cages where they could move freely and were allowed to recover for 90 min. After recovery, a blood sample was taken for measuring background enrichment of respective metabolites.

For measuring glucose production and glucose uptake rates under basal conditions, the animals were infused through the tail vein with a priming dose of 10 µCi [3-^{3}H]glucose/kg body wt followed by constant infusion of 0.33 µCi [3-^{3}H]glucose · min^{-1} · kg^{-1} body wt in saline for 70 min. Basal steady-state–specific activity of plasma glucose was determined in two blood samples drawn at 10-min intervals during the last 20 min of basal constant infusion. Clamped euglycemic hyperinsulinemia was induced by infusing human insulin (Actrapid, Novo, Denmark) at a rate of 8 mU · min^{-1} · kg^{-1} body wt for 120–130 min while maintaining euglycemia by infusion of 10% glucose in saline containing 4 µCi of [3-^{3}H]glucose/ml. Two to three blood samples were drawn at 10- to 20-min intervals during the last 40 min of euglycemic-hyperinsulinemic clamp and used for evaluating plasma glucose–specific activity under clamp conditions. Catheter patency was maintained by heparin. Sampled blood was replaced by replenishment with washed blood cells. Since blood replenishment requires the addition of heparin, which could interfere in measuring lipolytic fluxes, glycerol and fatty acid production rates were determined using a distinct experimental setup as follows:

For measuring fatty acids and glycerol production rates under basal conditions, the animals were constantly infused through the tail vein with 0.27 µmol [2,2-^{2}H]palmitate · min^{-1} · kg^{-1} body wt (bound to albumin at a ratio of 7:1) and 0.63 µmol [$^2H_5$]glycerol · min^{-1} · kg^{-1} body wt in saline for 150 min. Basal steady-state enrichment of plasma palmitate and glycerol was determined in three blood samples withdrawn at 10- to 20-min intervals during the last 60 min of basal constant infusion. Clamped euglycemic hyperinsulinemia was induced as described above while maintaining the infusion of [$^2H_2$]palmitate and [$^2H_5$]glycerol as described. Two to three blood samples were withdrawn at 10- to 20-min intervals during the last 60 min of euglycemic-hyperinsulinemic clamp and used for evaluating plasma palmitate and glycerol enrichment under clamp conditions. Catheter patency was maintained by saline to eliminate heparin during measurement of lipolytic rates. Total amount of blood sampled during these clamp studies was <5 ml, resulting in 15% overall decrease in hematocrit.

Plasma corticosterone level was monitored throughout clamp studies and found to remain unaffected under basal conditions (203 ± 35 vs. 270 ± 38 ng/ml in nontreated and MEDICA 16–treated animals, respectively) or clamped hyperinsulinemia (257 ± 11 vs. 272 ± 25 ng/ml in nontreated and MEDICA 16–treated animals, respectively).

Total body water. Total body water was measured by $H_2{}^{18}O$ dilution as described by (21,22) and modified by us for small blood samples. One hour before catheter placement, 0.35 g of $H_2{}^{18}O$ was injected and followed 90 min later by blood sampling. Oxygen 18 enrichment was analyzed using a triple inlet, triple collector isotope ratio mass spectrometer (SIRA II, V.G., U.K.). Body fat mass was evaluated as described by Klein et al. (22).

Oxygen consumption. Oxygen consumption was measured during the basal infusion step by placing the animals in a spacious perspex box for 30 min. O_2 consumption was measured using a NAGA oxygen analyzer (Franztec, Haifa, Israel).

Sample preparation for analyzing isotopic enrichment. Plasma [3-^{3}H]glucose–specific activity was determined by subjecting 100 µl plasma samples to barium hydroxide-zinc sulfate precipitation (Somogyi procedure), after which the supernatant was passed through a mixed cation/anion exchange resin.

Plasma [$^2H_2$]palmitate enrichment was determined by extracting 70 µl of plasma in 5 ml of 2N H_2SO_4:isopropanol:heptane 3:80:20 and followed by chromatographing the extract by thin-layer chromatography in heptane:diethyl-ether:glacial acetic acid 157:39:3.9. The purified fatty acids were derivatized to their methyl esters as described by Bier et al. (23). Fatty acyl methyl esters were subjected to gas chromatography–mass spectrometer (GC-MS) analysis.

Plasma [$^2H_5$]glycerol enrichment was determined by subjecting 150-µl plasma samples to barium hydroxide-zinc sulfate precipitation, after which the supernatant was passed through a mixed cation/anion exchange resin. The eluate was collected in reaction vials and evaporated to dryness. Glycerol was derivatized to its t-butyldimethylsilyl derivative essentially as described by Clouette (24). Briefly, 50–60 µl of anhydrous acetonitrile was added to the dried eluates, followed by the addition of 20 µl of the *N*-methyl-*N*(tertbutyldimethylsilyl)-trifluoroacetamide (MTB-STFA). The reaction vials were sealed with Teflon-lined caps, heated in a 110°C heating block for 15 min, and left to cool down for another hour at room temperature. t-Butyldimethylsilyl glycerol was subjected to GC-MS analysis.

GC-MS analysis. Isotopic enrichment was determined by GC-MS analysis using a Quatro II Fisons instrument quadruple mass spectrometer coupled to a gas chromatograph (15 m DB-1 GC capillary column, J & W Scientific, CA). The mass spectrometer was operated in the electron impact mode at an ionization energy of 70 eV and source temperature of 180°C. The mass spectrometer was daily tuned to the 219 and 264 m/e ions of heptacosa. Methylpalmitate enrichment was determined by selectively monitoring the m/e 270 (M) and 272 (M + 2) ions. t-Butyldimethylsilyl derivative of glycerol was determined by selectively monitoring the m/e of 217 (M) and 220 (M + 3) ions. The m/e 217 ion appeared to be the most prominent and stable fragment derived from t-butyldimethylsilyl glycerol. Selectively monitoring the m/e ions of 217 (M), and 220 (M + 3) provided an accurate measurement for plasma glycerol enrichment as verified by analyzing glycerol standards of varying glycerol enrichments.

Analytical procedures. Plasma insulin was measured by radioimmunoassay (Human Megenix, Belgium) using human insulin as standard. Plasma corticosterone was measured according to Weidenfeld et al. (25). Plasma glucose, triacylglycerol, and cholesterol were measured using commercial kits (Boehringer Mannheim). β-Hydroxybutyrate was measured in plasma samples deproteinized with perchloric acid using a commercial kit (Sigma, St. Louis, MO). Free fatty acid (FFA) composition of plasma was determined by GC analysis of methyl ester derivatives.

Calculations. Rates of appearance of palmitate (R_a palmitate) and glycerol (R_a glycerol) under basal or clamp conditions were calculated using Steele's equation for steady-state conditions (26), as modified by Bier (27) for stable isotopes:

$$R_a\ (\mu mol \cdot min^{-1} \cdot kg^{-1}) = \left[\left(\frac{IE_{in}}{IE_p}\right) - 1\right] \times F,$$

where F is the isotope infusion rate (micromoles per minute per kilogram), IE_{in} is the isotopic enrichment of the infusate, and IE_p is the isotopic enrichment of plasma at isotopic equilibrium expressed in mole percent excess. Rate of FFA appearance (R_a FFA) was calculated by dividing R_a palmitate by the ratio of palmitate to total FFA concentration in plasma.

The following intracellular reesterification rates were calculated as described by Wolfe et al. (28) and Campbel et al. (29).

$$\text{Intracellular reesterification rate } (\mu mol \cdot min^{-1} \cdot kg^{-1}) = \text{fatty acid production rate} - R_a \text{ FFA},$$

where the production rate of fatty acids equals 3 × R_a glycerol.

Glucose disposal rate was calculated using Steele's equation (26) accounting for added radiolabeled glucose entering the system along with the exogenous glucose infusate (30). Insulin metabolic clearance rate was calculated according to DeFronzo et al. (31).

Statistics. All values are means ± SE. Basal and insulin-clamped values were compared by a paired *t* test, while comparison between groups was made by unpaired *t* test.

RESULTS

Basal characteristics. The effect of MEDICA 16 treatment on concentrations of plasma insulin and metabolites in fed Zucker (*fa/fa*) obese rats is presented in Table 1. MEDICA 16 treatment resulted in a 15% reduction in plasma glucose with a concomitant 50% decrease in plasma insulin. Plasma triacylglycerols, cholesterol, and palmitate were reduced by 60, 40, and 40%, respectively, while plasma β-hydroxybutyrate concentration was increased 2.3-fold by MEDICA 16 treatment. The calorigenic activity of MEDICA 16 was exemplified by a 23% increase in oxygen consumption of treated animals (16.7 ± 0.7 vs. 13.6 ± 0.8 ml O_2 · min^{-1} · kg^{-1} body wt ($P < 0.05$)

TABLE 1
Plasma insulin and metabolites in fed MEDICA 16–treated Zucker rats

	Insulin (μU/ml)	Glucose (mg %)	Triacylglycerol (mg %)	Cholesterol (mg %)	Palmitate (μg/ml)	β-OH-butyrate (μmol/l)
Nontreated	146 ± 15	105 ± 7	379 ± 22	153 ± 10	59 ± 8	300 ± 24
MEDICA 16–treated	77 ± 6*	89 ± 2*	148 ± 12*	92 ± 11*	35 ± 5*	689 ± 41*

Data are means ± SE (n = 8). Zucker (*fa/fa*) rats were treated with MEDICA 16 as described in METHODS. Plasma metabolites were determined in tail blood samples of fed animals as described in METHODS. *Significantly different from respective nontreated value ($P < 0.05$, t test).

in MEDICA 16–treated and nontreated animals, respectively), compensated by a 6–8% increase in food consumption. Body weight gain as well as body composition determined by $H_2{}^{18}O$ dilution remained unaffected (41 ± 2 vs. 43 ± 3% fat content in nontreated and MEDICA 16–treated animals, respectively).

Insulin sensitivity. To evaluate the insulin-sensitizing activity of MEDICA 16 in vivo, the response to insulin was verified in postabsorptive animals under conditions of clamped euglycemic hyperinsulinemia as compared with basal conditions. The decrease in glucose, insulin, and plasma palmitate induced by MEDICA 16 treatment in the fed state (Table 1) was similarly observed in postabsorptive animals (Table 2, basal conditions). Sensitization to insulin was verified in terms of hepatic glucose production (HGP), total-body glucose disposal, and the lipolytic flux of fatty acids and glycerol in adipose tissue.

The effect of MEDICA 16 treatment on HGP and total-body glucose disposal rates is shown in Fig. 1. HGP and total-body glucose disposal rates in nontreated animals remained essentially unaffected by induced hyperinsulinemia, thus reflecting the severe resistance to insulin in liver and muscle tissue of the obese Zucker (*fa/fa*) rat. MEDICA 16 treatment did not result in decreasing hepatic glucose production or improving glucose disposal rates under basal conditions. However, in contrast to nontreated animals, treatment resulted in a pronounced sensitization to insulin with a 47% insulin-induced decrease in HGP (45 ± 2 vs. 24 ± 3 μmol · min^{-1} · kg^{-1}), together with a 64% insulin-induced increase in total-body glucose disposal rate (45 ± 2 vs. 74 ± 4 μmol · min^{-1} · kg^{-1}) under conditions of clamped euglycemic hyperinsulinemia. The insulin-sensitizing activity of MEDICA 16 in liver and muscle tissue could be further demonstrated by the 3.8-fold higher glucose infusion rate required to maintain euglycemia in treated insulin-infused animals as compared with nontreated animals (49 ± 5 vs. 13 ± 4 μmol glucose · min^{-1} · kg^{-1}, respectively).

It is noteworthy that the steady-state concentrations of plasma insulin attained with constant infusion of 8 mU of insulin · min^{-1} · kg^{-1} were 1.8-fold lower in MEDICA 16–treated animals (Table 2). Hence, the insulin-sensitizing activity of MEDICA 16 in liver and muscle tissue could be expected to be even more pronounced if evaluated under conditions of similar plasma insulin levels in nontreated and MEDICA 16–treated insulin-infused animals. This pronounced difference in plasma insulin levels between treated and nontreated animals under conditions of similar insulin infusion rates may be ascribed to a pronounced increase in metabolic clearance of plasma insulin induced by MEDICA 16 treatment (18.1 ± 1.8 vs. 32.1 ± 4.1 ml · min^{-1} · kg^{-1}, $P < 0.05$, respectively). A similar effect has been observed in lean as compared with obese Zucker rats (33) as well as in Zucker rats treated with a thiazolidinedione compound (38). This higher metabolic clearance of insulin may reflect an additional facet of liver sensitization to insulin.

Sensitization of adipose tissue to insulin by MEDICA 16 treatment was evaluated by studying fatty acids and glycerol fluxes in conscious unrestrained animals (32) under basal conditions, as compared with clamped euglycemic-hyperinsulinemic conditions using a modified and extended experimental design. As shown in Fig. 2, nontreated and MEDICA 16–treated animals attained steady-state enrichment of plasma glycerol and fatty acid under basal conditions as well as insulin clamp conditions. The basal flux rates of FFA (R_a FFA) and glycerol (R_a glycerol) remained unaffected by insulin infusion, indicating complete resistance to insulin in adipose tissue in a nontreated animal (Fig. 2A). However, insulin infusion resulted in a pronounced decrease in R_a FFA in MEDICA 16–treated animals (Fig. 2*B*). Figure 3 summarizes

TABLE 2
Plasma insulin and metabolites in postabsorptive MEDICA 16–treated Zucker rats

	Nontreated		MEDICA 16–treated	
	Basal conditions	Clamped euglycemic hyperinsulinemia	Basal conditions	Clamped euglycemic hyperinsulinemia
Plasma insulin (μU/ml)	233 ± 26	692 ± 60†	77 ± 7*	380 ± 41*†
Plasma glucose (mg %)	112 ± 4	111 ± 4	95 ± 4*	95 ± 4*
Plasma palmitate (μg/ml)	228 ± 14	177 ± 17	118 ± 10*	51 ± 7*†

Data are means ± SE (n = 5–12). Plasma metabolites were determined under basal and clamped euglycemic hyperinsulinemic conditions in postabsorptive nontreated and MEDICA 16–treated Zucker (*fa/fa*) rats as described in METHODS. *Significantly different from the respective nontreated value ($P < 0.05$, paired t test); †significantly different from the respective basal value ($P < 0.05$, paired t test).

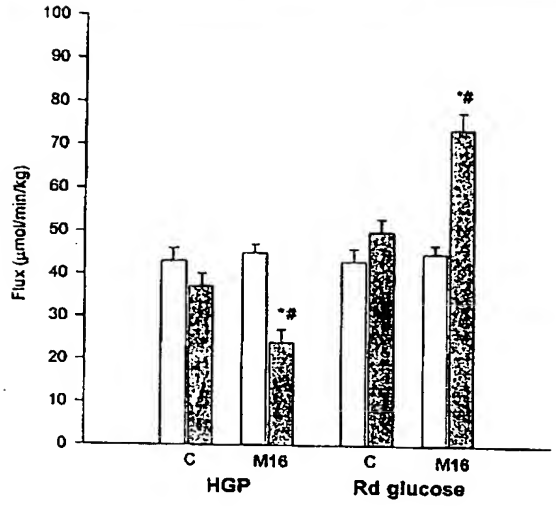

FIG. 1. HGP and glucose disposal rates in MEDICA 16–treated Zucker rats. HGP and glucose disposal rates (R_d glucose) were determined under basal (□) or clamped euglycemic hyperinsulinemic (▨) conditions in postabsorptive nontreated (C) and MEDICA 16-treated (M16) animals as described in METHODS. Mean + SE (n = 7–12). *Significantly different from the respective nontreated value (P < 0.05, paired t test); #significantly different from the respective basal value (P < 0.05, paired t test).

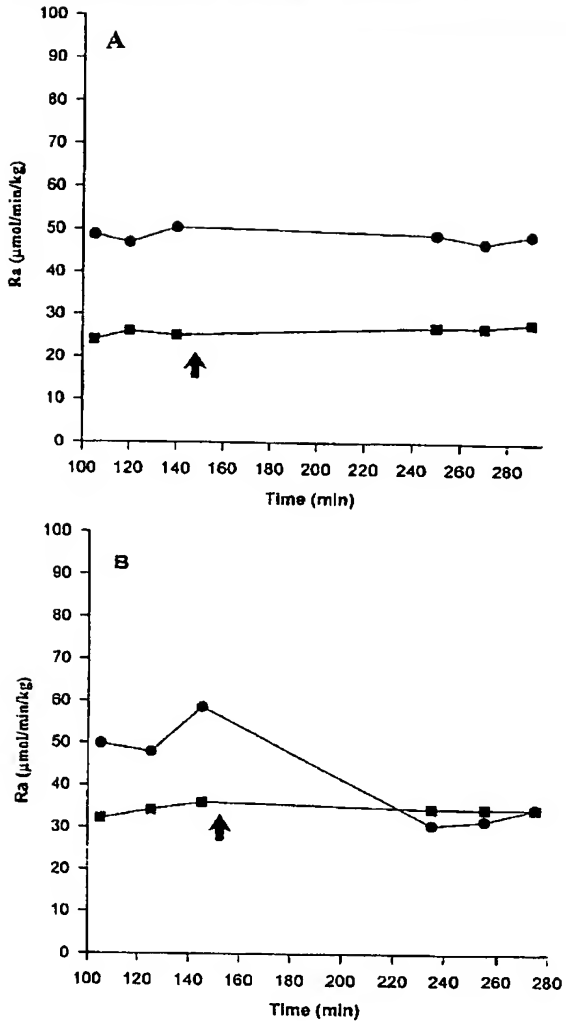

FIG. 2. FFA flux and glycerol flux in a nontreated and a MEDICA 16–treated Zucker rat. FFA flux (R_a FFA) (●) and glycerol flux (R_a glycerol) (■) were determined under basal or clamped euglycemic hyperinsulinemic conditions in a postabsorptive nontreated (A) and MEDICA 16–treated (B) animal as described. Arrow indicates the beginning of insulin infusion.

the basal and insulin-induced flux rates of FFA and glycerol for all clamped animals. Thus, insulin infusion resulted in a 35% inhibition of the basal flux rate of FFA in MEDICA 16–treated animals (55.2 ± 3.6 vs. 35.8 ± 2 μmol · min^{-1} · kg^{-1}), whereas R_a FFA remained essentially unaffected by insulin in nontreated rats. Inhibition of FFA flux rate by insulin in treated animals could not be accounted for by the production rate of FFA (3 × R_a glycerol) because basal R_a glycerol increased by 1.3-fold in treated animals and remained unaffected under clamped hyperinsulinemic conditions. Hence, the reduced FFA flux induced by insulin, in the face of increased FFA production rate in treated animals, had to be accounted for by a MEDICA 16–induced increase in the rate of reesterification of FFA in adipose tissue. Indeed, this was indicated by evaluating the reesterification rate of FFA (3 × R_a glycerol – R_a FFA) in nontreated and treated animals (Fig. 3) as well as the 1.5- to 2-fold decrease in the R_a FFA/R_a glycerol ratio in insulin-clamped MEDICA 16–treated animals as compared with nontreated animals (Fig. 4). Thus, 37% of the total FFA produced under basal conditions was intracellularly reesterified back into lipids in nontreated animals (28.2 ± 1.7 out of 76.2 ± 6.0 μmol · min^{-1} · kg^{-1}), and the extent of reesterification remained essentially unaffected by insulin. Basal intracellular reesterification of FFA was significantly increased in treated as compared with nontreated animals, amounting to 46% of the total FFA produced (47.3 ± 0.8 out of 102.0 ± 4.5 μmol · min^{-1} · kg^{-1}), and was further increased by insulin approaching 70% of total FFA produced (71.7 ± 3.5 out of 106.8 ± 1.8 μmol · min^{-1} · kg^{-1}). Thus, FFA reesterification flux in MEDICA 16–treated animals under clamped euglycemic-hyperinsulinemic conditions was ~2.5-fold higher than that of nontreated animals studied under basal or clamped conditions.

DISCUSSION

The obese Zucker (*fa/fa*) rat is a well-established animal model for insulin resistance (33,34). Insulin resistance has been verified here by evaluating the response to insulin of glucose production in the liver, total-body glucose disposal, and intracellular FFA cycling in adipose tissue under conditions of clamped euglycemic hyperinsulinemia. The obese Zucker (*fa/fa*) rat was indeed found here to maintain its basal values of HGP, total-body glucose disposal, and adipose tissue FFA cycling, irrespective of the induced insulin levels. Resistance to insulin was also reflected in basal hyperinsulinemia accompanied by hypertriglyceridemia, hypercholesterolemia, and increased plasma FFA. MEDICA 16 treatment resulted in sensitization to insulin, as verified by an insulin-induced decrease in HGP, together with an insulin-induced increase in total-body glucose disposal and, in particular, glucose dis-

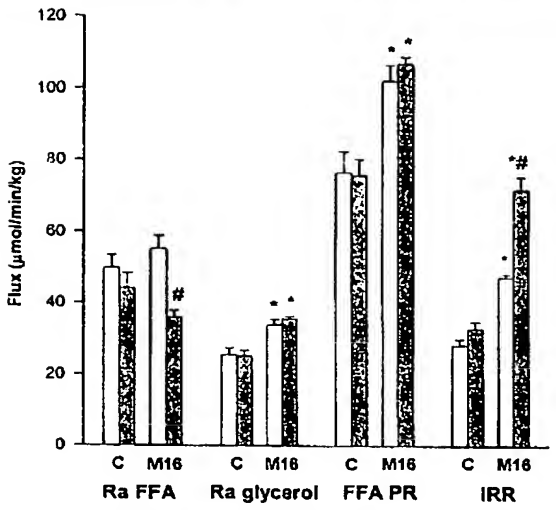

FIG. 3. FFA flux, glycerol flux, and FFA reesterification rates in MEDICA 16–treated Zucker rats. FFA flux (R_a FFA), glycerol flux (R_a glycerol), FFA production (FFA PR), and intracellular reesterification (IRR) rates were determined under basal (□) and clamped euglycemic-hyperinsulinemic (▨) conditions in postabsorptive nontreated (C) and MEDICA 16–treated (M16) animals as described in METHODS. Mean + SE (n = 3–5). *Significantly different from the respective nontreated value (P < 0.05, paired t test); #significantly different from the respective basal value (P < 0.05, paired t test).

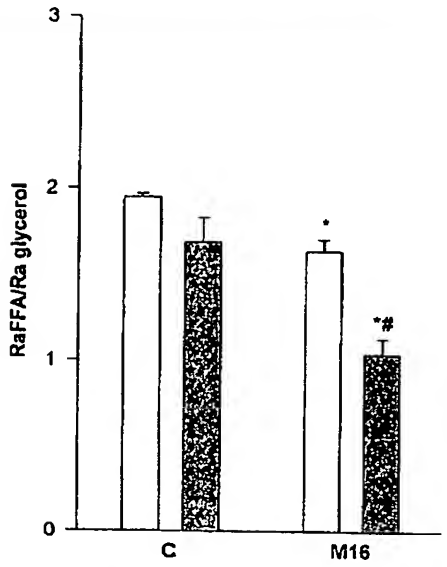

FIG. 4. FFA/glycerol flux ratio in MEDICA 16–treated Zucker rats. FFA flux (R_a FFA) and glycerol flux (R_a glycerol) were determined under basal (□) and clamped euglycemic hyperinsulinemic (▨) conditions in postabsorptive nontreated (C) and MEDICA 16–treated (M16) animals as described in METHODS. Mean + SE (n = 3–5). *Significantly different from the respective nontreated value (P < 0.05, paired t test); #significantly different from the respective basal value (P < 0.05, paired t test).

posed for FFA reesterification in adipose tissue. Glucose disposed for FFA reesterification as a fraction of total-body glucose disposal was estimated by the glycerol-3-phosphate required for maintaining insulin-induced FFA reesterification in MEDICA 16–treated as compared with nontreated animals (Table 3). A similar fraction of 13% of total-body glucose disposal was required for maintaining insulin-induced FFA reesterification both in treated as well as nontreated animals under conditions where insulin-induced FFA reesterification was 4.4-fold higher in the treated animals. Hence, sensitization of adipose tissue to insulin by MEDICA 16 was merely reflecting the overall sensitization to insulin induced by the drug. Sensitization to insulin by MEDICA 16 as reported here conforms our previous findings that pointed to an increase in the number of insulin receptors, together with a 5- to 6-fold increase in the basal activity of glycogen synthase in MEDICA 16–treated sand rats (17).

Sensitization to insulin by MEDICA 16 under conditions of clamped euglycemic hyperinsulinemia was only partially observed under basal conditions. Thus, MEDICA 16 treatment resulted in 1.7-fold activation of basal FFA reesterification in adipose tissue (47.3 ± 0.8 vs. 28.2 ± 1.7 μmol FFA · min^{-1} · kg^{-1} in treated and nontreated animals, respectively [Fig. 3]), whereas basal total-body glucose disposal or basal HGP remained unaffected by MEDICA 16 (Fig. 1). Basal sensitization to insulin of adipose tissue as contrasted with liver and muscle tissue may be accounted for by previous reports in Zucker rats that point to higher intrinsic sensitivity to insulin in adipose tissue as compared with liver and muscle tissue (34). The higher sensitivity of adipose tissue complemented by MEDICA 16–induced sensitization may more than compensate for the decreased basal plasma insulin levels induced by the drug. On the other hand, sensitization of liver and muscle tissue to insulin by MEDICA 16 could be compromised under basal conditions by the decreased plasma insulin levels induced by the drug. Hence, sensitization to insulin by MED-

TABLE 3
Insulin-induced glucose disposal in MEDICA 16–treated Zucker rats

	Nontreated	MEDICA 16–treated
Insulin-induced FFA reesterification rate	5.5 ± 2.8	24.4 ± 4.1*
Insulin-induced glucose conversion into glyceride-glycerol	0.9 ± 0.5	4.0 ± 0.7*
Insulin-induced total-body glucose disposal	7.0 ± 3.0	29.0 ± 5.0*

Data are means ± SE. Conditions as in Figs. 1 and 3. Insulin-induced rates (μmol · min^{-1} · kg^{-1}) were calculated by subtracting the basal values from the respective clamped euglycemic-hyperinsulinemic values. Glucose conversion rates into glyceride-glycerol were calculated from the respective FFA reesterification rates assuming three esterified residues of fatty acids/glyceride glycerol. *Significantly different from the respective nontreated value (P < 0.05, paired t test).

ICA 16 treatment could be expected to be even more pronounced if evaluated under conditions of similar plasma insulin levels in nontreated and MEDICA 16–treated animals.

Increased intracellular reesterification of FFA induced by MEDICA 16 in adipose tissue may account for the lower plasma levels of FFA under hyperinsulinemic conditions. Lowering of plasma FFA could be of crucial importance in maintaining sensitization to insulin in liver and muscle tissue (1–3), as well as in delaying fatty acid–induced pancreatic lipotoxicity and regression of impaired glucose tolerance to NIDDM (35). It is noteworthy, however, that adipose sensitization to insulin may eventually result in increased fat content, compromising the decrease in FFA efflux from adipose tissue induced by the drug. Hence, sustained pharmacological sensitization to insulin must be accompanied by sustained pharmacological calorigenic activity, which may counteract and prevent an increase in adipose mass under conditions of whole-body sensitization to insulin. Thus, the calorigenic activity of MEDICA 16 ([12–16], O. Hermesh, B.K., J.B.-T., unpublished observations), as verified here by increased oxygen consumption and ketogenesis as well as maintenance of body composition similar to that of nontreated animals in the face of induced sensitization of adipose tissue to insulin, is essential for its insulin-sensitizing activity. Caloric restriction of obese Zucker (*fa/fa*) rats may offer an alternative mode for maintaining body composition under conditions of sensitization to insulin (36). Hence, insulin sensitizers capable of inducing calorigenesis should be preferred for treating insulin resistance in general and obese individuals in particular.

Furthermore, liver sensitization to insulin by MEDICA 16 in the face of increased calorigenesis (12,15), hepatic fatty acid oxidation (14), and ketogenesis induced by the drug may indicate that sensitization to insulin is not mutually exclusive with increased fatty acid oxidation. While this notion still remains to be verified by evaluating fuel selection by extrahepatic glucose utilizing tissues in MEDICA 16–treated animals, studies concerned with Pima Indians have similarly indicated that NIDDM could be predicted by increased plasma FFA levels, but not by the extent of basal lipid oxidation (37). Hence, insulin resistance induced by increased plasma FFA levels may reflect direct suppression of limiting steps of glucose metabolism by the FFA or its immediate CoA-thioester rather than being ascribed to downstream β-oxidized metabolites of fatty acid oxidation (2). Sensitization to insulin by MEDICA 16 could result from the induced decrease in plasma FFA levels, complemented perhaps by the displacement of inhibitory fatty acids from their respective target sites by the nonproductive fatty acyl analog.

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EXHIBIT E

Applicants: Jacob Bar-Tana
Serial No.: 10/735,439
Filed: December 11, 2003

Exhibit E 4.8 ©

Original Articles

Hypolipidemic, Antiobesity, and Hypoglycemic-Hypoinsulinemic Effects of β,β′-Methyl–Substituted Hexadecanedioic Acid in Sand Rats

RUTH TZUR, GENE ROSE-KAHN, JONATHAN H. ADLER, AND JACOB BAR-TANA

Treatment of male sand rats kept on a balanced laboratory chow diet ad libitum with β,β′-tetramethyl–substituted hexadecanedioic acid (MEDICA 16) resulted in a hypolipidemic effect accompanied by an extensive reduction in adiposity, with a concomitant hypoglycemic-hypoinsulinemic effect. The overall effect was sustained as long as the drug was administered. The hypolipidemic effect of MEDICA 16 consisted of a 70 and 40% decrease in plasma triacylglycerols and cholesterol, respectively, and resulted from inhibition of liver lipogenesis and cholesterogenesis. Adipose reduction by MEDICA 16 treatment or calorie restriction consisted of a 75–90% decrease in the perirenal, omental, epididymal, and subcutaneous fat, with a 50% decrease in liver neutral lipids. The reduction in adiposity was accounted for by a respective decrease in the lipid content of individual adipocytes, with a concomitant decrease in the number of adipocytes of selected adipose tissues. The decrease induced in adiposity by MEDICA 16 treatment could not be accounted for by anorectic or cathartic effects of the drug. The hypoglycemic-hypoinsulinemic effect of MEDICA 16 consisted of amelioration of the tolerance of glucose with normalization of plasma insulin. It was accompanied by an eightfold increase in the number of insulin receptors in epididymal adipocytes, which was, however, counteracted by a decrease in their affinity for insulin. The receptor and postreceptor effects exerted by MEDICA 16 were similar to those of calorie restriction. The overall effect of MEDICA 16 in sand rats may reflect the pharmacological potential of MEDICA compounds in pathological hyperlipidemic-obesity-diabetic syndromes. *Diabetes* 37:1618–24, 1988

Long-chain fatty acids and their respective thioesters have repeatedly been reported to affect lipid metabolism by modulating key steps in lipid and lipoprotein synthesis and degradation. The modulating capacity of long-chain fatty acids, as opposed to their role as substrates, has initiated the design of nonmetabolic long-chain fatty acyl analogues to be exploited as hypolipidemic-antiobesity agents. β,β′-Methyl–substituted dicarboxylic acids (MEDICA) of C_{14}–C_{18} chain length [HOOC-CH_2-$C(CH_3)_2$-$(CH_2)_n$-$C(CH_3)_2$-CH_2-COOH, n = 8–12] appear to fulfill this role, with MEDICA 16 (n = 10) being the most potent of the concerned homologous series (1). Thus, the ω-carboxyl function interferes with the esterification of the dioic acid into lipid while still allowing for an ATP-dependent coenzyme A (CoA) thioesterification at either carboxylic end, and the β,β′-substitution prevents the β-oxidative catabolism of MEDICA compounds by either mitochondrial or peroxisomal systems. As a hypolipidemic drug in the rat, MEDICA 16 was found to potently inhibit liver ATP-citrate lyase, with a concomitant 80% inhibition of liver lipogenesis and cholesterogenesis (2). Inhibition of liver lipid synthesis resulted in a 60–70% decrease in plasma very-low-density lipoprotein (VLDL)-triacylglycerol and a 40–50% decrease in plasma VLDL-cholesterol under conditions of fat-free carbohydrate-rich feeding, where liver lipogenesis and cholesterogenesis constitute a major flux of liver lipid synthesis (1). MEDICA 16 was also found to act as a hypolipidemic effector under conditions of a balanced diet, which still allows for the production of lipoproteins from exogenous fatty acids and cholesterol (3,4). The 70% decrease in plasma chylomicrons-triacylglycerol observed under these conditions could be accounted for by an enhanced plasma catabolism of the triacylglycerol-rich lipoproteins due to a pronounced decrease in plasma apolipoprotein C III (3,4).

In the light of these features of MEDICA compounds, it became of interest to evaluate their potential as antiobesity agents in vivo in an animal model for obesity. The sand rat

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(*Psammomys obesus*) is the model of choice because of its spontaneous obesity and obesity-induced diabetes (5–12).

MATERIALS AND METHODS

Weaned male sand rats of the Hebrew University strain (13), weighing 66 ± 16 g, were either maintained on a laboratory chow diet (Amrod 935, Ambar, Hadera, Israel) fed ad libitum in the presence or absence of added 0.1% (wt/wt) of MEDICA 16 or were calorie restricted by being maintained on 4 g Amrod 935 per day supplemented ad libitum with fresh salt bush (*Atriplex halimus*) leaves. The Amrod 935 chow consisted of 11.7% (wt/wt) moisture, 50.4% (wt/wt) nitrogen-free extract (carbohydrate), 17.3% (wt/wt) protein, 4.0% (wt/wt) fat, 8.3% (wt/wt) fiber, and 8.1% (wt/wt) ash. The fresh salt bush consisted of 61.5% (wt/wt) moisture, 15.2% (wt/wt) nitrogen-free extract (carbohydrate), 5.0% (wt/wt) protein, 0.7% (wt/wt) fat, 9.9% (wt/wt) fiber, and 8.9% (wt/wt) ash. The animals were supplied with water ad libitum. The ad libitum chow consumption ranged from 10 ± 1 to 15 ± 1 g/day as a function of age. Animals maintained on calorie-restricted diet consumed 10–20 g/day of fresh salt bush leaves as a function of age. The overall calorie consumption of calorie-restricted animals was ~75% of the ad libitum chow ration. MEDICA 16 was administered by soaking the diet pellets in an ether solution of MEDICA 16 followed by exhaustive flash evaporation of the solvent.

Glucose tolerance was determined in nonfasting rats lightly anesthetized with 1% phenobarbital and subsequently injected with 100 mg glucose/100 g body wt i.p. in saline. Body temperature was maintained at 37°C by using heat lamps. Blood samples were collected from the retro-orbital venous plexus in heparin-coated capillary tubes 0, 1, 2, and 3 h after glucose loading. The blood samples were immediately cooled, centrifuged at 4°C, and plasma glucose was determined by the glucose oxidase method.

Plasma insulin, plasma glucose, and plasma lipids were determined in tail vein blood collected into heparinized capillary tubes or in blood from the abdominal vena cava of ether-anesthetized animals collected after they were killed. Plasma insulin was determined by a magnetic antibody immunoassay kit (Insulin Maia Kit, Serono, Coinsins, Switzerland). Plasma triacylglycerols and plasma cholesterol were determined enzymatically using Boehringer (Mannheim, FRG) kits 244473 and 172626, respectively.

^3H_2O incorporation into liver lipid in vivo was determined as previously described (1). Liver citrate, malonyl-CoA, and acetyl-CoA were determined as previously described (1).

Epididymal, perirenal, and omental adipocytes were prepared by collagenase type II treatment as described by Rodbell (14). To minimize cell disruption the tissues were digested for short incubation periods under conditions of gentle shaking. The diameter of the isolated fat cells was measured microscopically at ×400 with a micrometer fixed into the microscope eyepiece. Five replicate aliquot samples were used for each fat cell preparation, and ~100 cells were screened in each aliquot sample to yield the mean diameter ± SD for each adipocyte preparation. The lipid content per fat cell for each adipocyte preparation was calculated by regarding the fat cell as a sphere with a volume as determined above and filled with tripalmitin (density 0.86 g/ml). The number of adipocytes per adipose tissue was calculated by dividing the total lipid ester content of the tissue by the lipid content per fat cell (15). The total lipid ester content was determined by hydroxamate formation (16).

Insulin binding to epididymal adipocytes was determined under equilibrium conditions essentially as described by Gammeltoft and Gliemann (17). Thus, 2×10^5 cells were incubated in duplicate in 500 µl of Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) containing 20 mg/ml bovine serum albumin, 0.1 µCi [^{125}I]monoiodoinsulin (Nuclear Research Center, Negev, Israel), and 0.5–160 ng unlabele porcine insulin. After incubation for 45 min at 37°C, a 200-µl aliquot of the cell suspension was transferred in duplicate to a plastic microtube containing 100 µl silicone oil (type 350, Merck, Darmstadt, FRG; sp gr 0.97 g/ml) and was centrifuged at maximal speed for 40–60 s in a Beckman 152 microfuge. The microtube was then cut through the silicone oil layer and the top and bottom layers were counted for radioactivity. Nonspecific binding was determined in the presence of an excess of unlabeled porcine insulin and was subtracted from the total bound count to yield the specific binding of insulin. Generally, the bound/free versus bound Scatchard plots were curvilinear, having an upward concavity with clearly distinguished high- and low-affinity sections. The apparent dissociation constant and the number of insulin receptors were derived by considering the high-affinity section of the Scatchard plot.

Glucose incorporation into adipose glycogen was determined by incubating a weighted piece (~150 mg) of the interscapular adipose tissue in 1.0 ml of KRB buffer (pH 7.4) containing 16 mM of [U-^{14}C]-D-glucose (New England Nuclear, Boston, MA; sp act 0.625 Ci/mol) in the presence or absence of porcine insulin. After incubation for 3 h at 37°C the tissue was washed in KRB buffer, dried, and extracted in isopropanol/heptane/1 N H_2SO_4 (40:10:2). The lipid-de-

TABLE 1
Antilipogenic-hypolipidemic effect of MEDICA 16 in sand rats fed ad libitum

	Untreated	MEDICA 16–treated
Plasma triacylglycerol (mg/dl)	205.0 ± 29.0 (6)	62.0 ± 15.0 (7)
Plasma cholesterol (mg/dl)	69.0 ± 7.0 (6)	39.6 ± 7.0 (7)
Liver lipids (µeq/g)	224.0 ± 24.0 (6)	133.0 ± 13.0 (7)
^3H_2O incorporation into liver lipids (µmol $^3H_2O \cdot g^{-1} \cdot 120\ min^{-1}$)		
Triacylglycerols	16.3 ± 4.4 (4)	5.1 ± 2.0 (4)
Phospholipids	4.4 ± 1.0 (4)	8.3 ± 1.9 (4)
3-β-Hydroxysterols	1.4 ± 0.2 (4)	0.4 ± 0.1 (4)
Total lipids	22.8 ± 4.4 (4)	14.5 ± 2.7 (4)
Liver citrate (nmol/g)	174.0 ± 25.0 (3)	69.5 ± 3.5 (3)
Liver acetyl-CoA (nmol/g)	132.0 ± 30.0 (3)	35.5 ± 8.0 (3)
Liver malonyl-CoA (nmol/g)	20.0 ± 3.0 (3)	5.0 ± 3.0 (3)

Values are means ± SD with number of animals in parentheses. Weaned male sand rats were treated by 0.1% (wt/wt) MEDICA 16 for 140 days as described in MATERIALS AND METHODS. Plasma triacylglycerol, plasma cholesterol, liver lipids, liver intermediate metabolites, and the incorporation of ^3H_2O into liver lipids were determined as described in MATERIALS AND METHODS. Liver weight at death was 7.6 ± 0.5 g (n = 6; 3.7% body wt) and 10.9 ± 2.0 g (n = 7; 7.0% body wt) for untreated and MEDICA 16–treated rats, respectively. All MEDICA 16–treated values significantly different ($P < .01$) from respective values of untreated rats.

TABLE 2
Adipose reduction by MEDICA 16 in sand rats

	Epididymal fat			Perirenal fat			Omental fat		
	Untreated	Calorie restricted	MEDICA 16 treated	Untreated	Calorie restricted	MEDICA 16 treated	Untreated	Calorie restricted	MEDICA 16 treated
Tissue weight (g)	3.8 ± 1.4	1.3 ± 0.3*	1.2 ± 0.5*	2.7 ± 1.3	0.7 ± 0.9*	0.5 ± 0.2*	1.7 ± 0.5	0.3 ± 0.1*	0.2 ± 0.1*
Tissue lipid content (mg/tissue)	1840 ± 583	522 ± 158*	315 ± 139*	1004 ± 601	273 ± 82*	85 ± 52*	449 ± 144	69 ± 39*	30 ± 20*
Adipocyte lipid content (μg/cell)	0.49 ± 0.13	0.15 ± 0.03*	0.13 ± 0.05*	0.66 ± 0.23	0.26 ± 0.04*	0.22 ± 0.05*	0.49 ± 0.28	0.17 ± 0.03*	0.11 ± 0.03*
Tissue cell number ($\times 10^{-6}$)	3.9 ± 1.2	3.5 ± 0.8	3.1 ± 1.3	1.5 ± 0.6	1.0 ± 0.2	0.4 ± 0.0*	1.1 ± 0.6	0.4 ± 0.2*	0.2 ± 0.2*

Values are means ± SD. Weaned male sand rats were maintained for 90–95 days on Amrod 935 fed ad libitum in the presence (n = 17) or absence (n = 19) of 0.1% (wt/wt) MEDICA 16 or were calorie restricted (n = 17) as described in MATERIALS AND METHODS. Total lipid content, adipocyte lipid content, and tissue cell number were determined for the epididymal, perirenal, and omental fats as described in MATERIALS AND METHODS. Tissue weight, tissue lipid content, adipocyte lipid content, and tissue cell number of the adipose tissues are combined from right and left pads.
*Significantly different ($P < .01$) from respective untreated value.

pleted tissue was then digested in 33% of boiling KOH for 20 min, and glycogen was precipitated by absolute ethanol at −20°C as described by Gutman et al. (18). The precipitate was dissolved in 0.5 ml H_2O and was counted in 40% Lumax in toluene.

Significance was analyzed by the Mann-Whitney U test.

MEDICA 16 was synthesized as previously described (1). Collagenase type II was from Sigma (St. Louis, MO). Crystalline porcine insulin was provided by Lilly (Indianapolis, IN).

RESULTS

Hypolipidemic effect. The overall hypolipidemic effect of MEDICA 16 in sand rats was essentially similar to that previously observed in albino rats (1,3) and consisted of a 70 and 40% decrease in total plasma triacylglycerol and cholesterol, respectively (Table 1). The observed hypolipidemic effect could be accounted for by 70% inhibition of liver lipogenesis and cholesterogenesis as determined by the incorporation of ^3H_2O into liver triacyglycerol–fatty acids and liver 3-β-hydroxysterols (Table 1), whereas the esterification of glycerol into neutral lipids in the presence of added palmitate remained unaffected (not shown). Inhibition of liver lipogenesis and cholesterogenesis could be ascribed to inhibition of liver ATP-citrate lyase (2) with a concomitant drastic reduction in liver acetyl-CoA and malonyl-CoA content (Table 1). However, in contrast with albino rats, where the content of liver citrate remained essentially unaffected under conditions of treatment with MEDICA 16 (1), the content of liver citrate in MEDICA 16–treated sand rats decreased twofold, although less remarkably than that of acetyl-CoA and malonyl-CoA. It is worth noting that the decrease in liver triacylglycerol and 3-β-hydroxysterol synthesis in MEDICA 16–treated sand rats was quite pronounced even when calculated on the basis of whole-liver fluxes, and in spite of the significant increase in liver weight of treated animals (Table 1).

Antiobesity effect. Treatment of sand rats fed ad libitum by MEDICA 16 resulted in an extensive decrease in the content of neutral lipids of the epididymal, perirenal, and omental fats (Table 2). Adipose reduction in MEDICA 16–treated sand rats was similar to that of calorie-restricted animals maintained on 70–80% of their ad libitum calorie ration. Thus, the final reduction in fat in MEDICA 16–treated rats amounted to 75, 92, and 93% for the epididymal, perirenal, and omental fats, respectively. In many cases the omental fat could hardly be detected in MEDICA 16–treated animals, whereas it could always be definitely recognized under conditions of calorie restriction. Adipose reduction by MEDICA 16 was primarily due to a 60–80% decrease in the neutral lipid content of individual adipocytes of the three adipose tissues studied, whereas the cell number was selectively affected. Thus, the perirenal and omental fats appeared to lose ~75% of their mature adipocytes in the course of MEDICA 16 treatment, whereas the number of cells in the epididymal fat remained essentially unaffected. Hence, the decrease in total lipid of the perirenal and omental fats could be accounted for by a decrease in the cellular lipid content of individual adipocytes as well as in the cell number of both tissues. On the other hand, the decrease in total lipid of the epididymal fat was not accompanied by a significant decrease in the number of epididymal fat cells and could be totally ascribed to the depletion of intracellular fat. The reduction in adiposity of MEDICA 16–treated sand rats was not only confined to the adipose tissues described but included subcutaneous adipose tissues (not shown) as well as fat deposits within splanchic organs, e.g., liver (Table 1).

Adipose reduction by MEDICA 16 was accompanied by a respective decrease in body weight that could be accounted for by the body composition of sand rats (13) and the antiobesity effect of the drug (Table 3). The weight-reductive effect of MEDICA 16 was somewhat more extensive than that effected by calorie restriction. The decrease in adiposity and body weight of MEDICA 16–treated sand rats could not be ascribed to a decrease in the net calorie intake as a result of either anorectic or cathartic effects of the drug. Thus, the daily ad libitum food consumption of MEDICA 16–treated sand rats was similar to that of untreated age-

TABLE 3
Weight reduction by MEDICA 16 in sand rats

Weight (g)	Untreated	Calorie restricted	MEDICA 16 treated
Initial	62 ± 11	66 ± 16	69 ± 16
Final	195 ± 16	171 ± 13	161 ± 14*
Gain	133 ± 19	105 ± 11	92 ± 18*

Values are means ± SD. Conditions as in Table 2.
*Significantly different ($P < .01$) from respective untreated value.

TABLE 4
Effect of MEDICA 16 on plasma glucose and insulin in sand rats

	Untreated	Calorie restricted	MEDICA 16 treated
Plasma glucose (mg/dl)	126 ± 38	95 ± 25	90 ± 18
Plasma insulin (μU/ml)	220 ± 63	52 ± 16*	48 ± 15*

Values are means ± SD. Conditions as in Table 2. Plasma glucose and insulin concentrations were determined as described in MATERIALS AND METHODS.
*Significantly different ($P < .01$) from respective untreated value.

matched animals kept under the conditions described in Tables 2 and 3 (10 ± 1 g chow/day for weaned animals weighing 70 g; 15 ± 1 g chow/day for older animals weighing 150–200 g). Similarly, the consistency of stool remained unaffected by MEDICA 16 treatment. Thus, the decrease in adiposity appeared to reflect a metabolic modality rather than a decrease in net calorie intake.

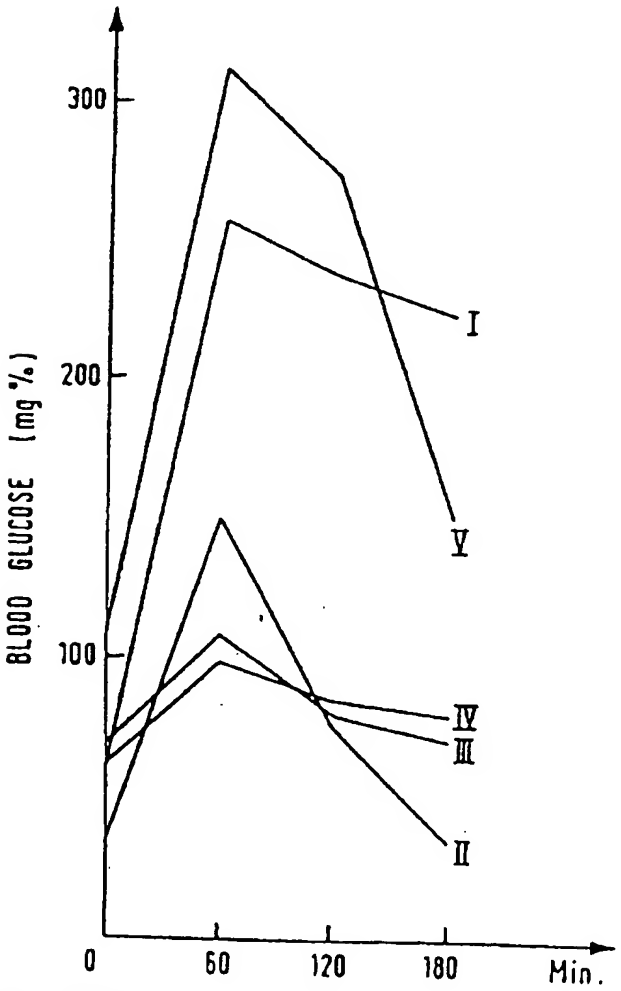

FIG. 1. Glucose tolerance in MEDICA 16–treated sand rats. Conditions as in Table 5. Glucose tolerance curves presented are those obtained with one representative animal (rat 3 in Table 5). I, pretreatment; II, 27th day of MEDICA 16 treatment; III, 71st day of MEDICA 16 treatment; IV, 34th day after cessation of MEDICA 16 treatment; V, 53rd day after cessation of MEDICA 16 treatment.

Hypoglycemic-hypoinsulinemic effect. MEDICA 16 treatment of sand rats maintained on laboratory chow ad libitum resulted in some decrease in plasma glucose, with a concomitant significant decrease in plasma insulin (Table 4). The plasma insulin level approached that of calorie-restricted animals and was in the range of that observed in normal albino rats of the Hebrew University strain. Moreover, the plasma insulin values in MEDICA 16–treated or calorie-restricted animals were within a limited range of 20–60 μU/ml, whereas the plasma insulin prevailing in untreated sand rats fed ad libitum varied within a broad range of 100–500 μU/ml.

Sand rats used in this study could be divided into two subgroups with respect to their glucose tolerance in response to an intraperitoneal glucose load. In ~75% of the animals the sum of the glucose values determined at 0, 1, 2, and 3 h after the injection of 100 mg glucose/100 g body wt i.p. was <400 mg/dl, in the range of that of calorie-restricted animals, whereas in ~25% of the population the sum amounted to 700–1000 mg/dl, thus defining a pathological tolerance pattern. The tolerance of glucose in sand rats selected for their decreased glucose tolerance was dramatically improved by MEDICA 16 treatment (Fig. 1; Table 5). Thus, as shown for the individual case of Fig. 1, the pathological glucose tolerance pattern of the untreated animal reverted back to normal after 27 days of treatment, and the normal pattern was sustained as long as treatment was maintained as well as during a 1-mo period after the suspension of the drug from the diet (Fig. 1; Table 5). In contrast to the improved glucose tolerance in sand rats treated by MEDICA 16, streptozocin-induced hyperglycemia in albino rats could not be improved by MEDICA 16 treatment. Thus, the plasma glucose in untreated streptozocin-induced diabetic and MEDICA 16–treated albino rats amounted to 610 ± 46 and 578 ± 13 mg/dl (means ± SD; $n = 6$), respectively, after 1 wk of treatment with 0.25% (wt/wt) of MEDICA 16 in the diet. Hence, the improved performance in MEDICA 16–treated sand rats required the presence of insulin and had to be ascribed to an improved peripheral handling of the glucose load.

The putative decrease in the peripheral resistance to insulin in MEDICA 16–treated sand rats was assessed by evaluating the number of insulin receptors in epididymal adi-

TABLE 5
Glucose tolerance in MEDICA 16–treated sand rats

Rat no.	Pretreatment	Treatment	Posttreatment
1	779, 1082	364	
2	947, 980, 958	442	
3	790	296, 329, 326	849
4	992	358, 336, 465	1049
5	867	434, 257, 388	470
6	717	555, 555	

Values are in milligrams per deciliter. Six sand rats were selected out of 25 animals for their distinctive pathological glucose tolerance and were treated with 0.1% (wt/wt) MEDICA 16 for 71 days. Pretreatment and treatment periods were followed by normal Amrod 935 diet (posttreatment). Each glucose tolerance test is represented by sum of glucose values (mg/dl) determined at 0, 1, 2, and 3 h. Duplicate or triplicate numbers refer to repeated glucose tolerance tests made during pretreatment or treatment periods.

TABLE 6
Epididymal insulin receptors in MEDICA 16–treated sand rats

	Untreated	Calorie restricted	MEDICA 16 treated
Insulin receptors/cell ($\times 10^{-4}$)	0.3 ± 0.1 (9)	2.4 ± 0.4 (11)*	2.6 ± 0.6 (8)*
K_a (M $\times$ 10^9)	0.4 ± 0.0	3.2 ± 0.8*	3.8 ± 0.3*

Values are means ± SD with number of animals in parentheses. Conditions as in Table 2.
*Significantly different ($P < .01$) from respective untreated value.

pocytes of untreated and MEDICA 16–treated animals. As shown in Table 6, MEDICA 16 treatment and calorie restriction resulted in eight- to ninefold increase in the number of insulin receptors per cell. The increase in insulin receptors effected by either MEDICA 16 or calorie restriction was, however, counteracted by a decrease in their affinity for insulin (Table 6).

Discrete postreceptor effects of MEDICA 16 were investigated by studying the incorporation of glucose into glycogen in the interscapular adipose tissue of sand rats maintained under the three intervention modes used (Table 7). Even in the absence of added insulin, the overall incorporation of glucose into adipose glycogen was increased fivefold, both in calorie-restricted and MEDICA 16–treated animals, compared with that of untreated rats fed ad libitum. Insulin was found to increase the incorporation rate of glucose by 20% in the treated and calorie-restricted animals, whereas it was essentially ineffective in untreated sand rats fed ad libitum.

DISCUSSION

Treatment of male sand rats kept on a balanced chow diet ad libitum with MEDICA 16 serving as a model compound for substituted long-chain β,β-dicarboxylic acids resulted in an extensive hypolipidemic effect accompanied by a reduction in adiposity, with a concomitant hypoglycemic-hypoinsulinemic effect. The overall effect was sustained as long as the drug was administered.

The hypolipidemic effect of MEDICA 16 in sand rats appears to be essentially similar to that reported in albino rats (1–3). Thus, in both species the hypolipidemic effect could be accounted for by inhibition of liver lipogenesis and cholesterogenesis as inferred from the incorporation of ^3H_2O into liver triacylglycerol–fatty acids and 3-β-hydroxysterol. Because the inhibition of the two synthetic pathways by MEDICA 16 was similar, it could be ascribed to inhibition of a metabolic step common to both. In the light of the established inhibition of liver ATP-citrate lyase by MEDICA 16 (2) and in line with the fourfold decrease in liver acetyl-CoA and malonyl-CoA content reported herein, the inhibition of liver lipogenesis and cholesterogenesis in MEDICA 16–treated sand rats is presumably accounted for by a crossover point at the ATP-citrate lyase step resulting in limitation of cytosolic acetyl-CoA for liver lipid synthesis.

The liver weight of MEDICA 16–treated sand rats was remarkably increased compared with that of untreated animals, in terms of absolute weight and relative to body weight (Table 1). The increase in liver weight was similar to that previously observed in albino rats treated with MEDICA 16 (19) and reflects the peroxisome proliferative capacity of the drug in rodents (19,20). The hypertrophic-hyperplastic effect initiated by MEDICA 16 acting as a peroxisome proliferator may also account for the observed increase in the relative amount of fatty acids channeled into phospholipids (21). Thus, the lipogenic flux culminating in phospholipids approached values of 19 and 57% of the total lipogenic flux in untreated and MEDICA 16–treated sand rats, respectively (Table 1).

The reduction in adipose fat in MEDICA 16–treated sand rats was characterized by its extensive scope and specificity with respect to the adipose tissues affected. Adipose reduction by MEDICA 16 was reversible, and elimination of the drug from the diet resulted in a rapid gain of adipose fat (R.T., unpublished observations). Because in sand rats, as opposed to albino rats, adipose tissue lipogenesis plays only a minor role in the overall synthesis of adipose fat (22), the inhibition of liver lipogenesis by MEDICA 16 presumably plays a causal role in adipose fat reduction by MEDICA 16. Furthermore, because adipose fat storage in sand rats is associated so much with liver triacylglycerol secretion (8) and the availability of plasma lipoproteins (22), the hypolipidemic effect induced by MEDICA 16 may be realized as the direct etiological cause for the adipose fat reduction observed. Moreover, as the K_m value of adipose lipoprotein lipase for plasma triacylglycerol is ~0.7 mM (23) and because the triacylglycerol concentrations prevailing in untreated and treated sand rats (2 and 0.7 mM, respectively) are in the range of the K_m value, the intravascular hydrolysis of plasma triacylglycerol by adipose lipoprotein lipase proceeds close to a first-order rate within the concerned range

TABLE 7
Glucose incorporation into adipose glycogen in MEDICA 16–treated sand rats

	Untreated	Calorie restricted	MEDICA 16 treated
Without insulin	208 ± 117 (3)	971 ± 512 (4)*	1117 ± 730 (5)*
With insulin	223 ± 79 (3)	1162 ± 833 (4)*†	1408 ± 860 (5)*†

Values are ng glucose · g^{-1} tissue · 3 h^{-1} (means ± SD), with number of animals in parentheses. Conditions as in Table 2, with and without 50 ng added insulin.
*Significantly different ($P < .01$) from respective untreated value.
†Significantly different ($P < .05$) from respective value without insulin.

of plasma triacylglycerol. Hence, the threefold decrease effected by MEDICA 16 in plasma triacylglycerol is expected to result in a respective decrease in the uptake of plasma lipids for adipose storage. It is worth noting that the overall activity of adipose lipoprotein lipase was found to remain unaffected in MEDICA 16–treated albino rats (R.T., unpublished observations).

The overall reduction effected by MEDICA 16 in adipose fat in the absence of either a concomitant decrease in net calorie intake or fat accumulation within splanchnic organs can only be made possible by expenditure of the calories spared from storage in adipose tissue. Indeed, MEDICA 16 treatment was recently observed to induce a 1.4-fold increase in the resting metabolic rate of albino rats (R.T., E. Smith, J.B.-T., unpublished observations), which may account for the overall energy expenditure of MEDICA 16–treated animals. This observed increase in basal oxygen consumption corroborates our previous results in which the irreversible disposal rate of glucose into carbon dioxide was found to be increased 1.3-fold in MEDICA 16–treated rats, and the observed increase could account for the glucose carbons spared from net lipid synthesis in the treated animals (1). The mechanism responsible for the MEDICA 16–induced increase in energy expenditure remains to be investigated.

Adipose reduction induced in sand rats by either MEDICA 16 treatment or calorie restriction differs from that induced by calorie restriction in albino rats with respect to the changes observed in the number of adipocytes of selected adipose tissues. Thus, calorie restriction of albino rats was repeatedly reported to leave the number of gonadal, inguinal, retroperitoneal, and subcutaneous adipocytes unchanged and to affect only their cellular lipid content (24,25). Similarly, the number of total adipocytes in lean sand rats was previously reported to remain unchanged under conditions of calorie-restriction–induced reduction in the total dissectable fat of obese animals (26). The epididymal tissue of our sand rats appears to follow the albino rat example (Table 2). On the other hand, the number of omental adipocytes was observed here to be significantly reduced by calorie restriction or MEDICA 16 treatment, and MEDICA 16 treatment also resulted in a significant and prompt reduction in the number of retroperitoneal adipocytes (Table 2). It is worth noting that the reduction in omental adipocytes observed here under conditions of calorie restriction could not be previously detected because the total dissectable fat rather than individual adipose tissues was subjected to analysis (26), and the mesenteric fat constitutes only a minor portion of the overall dissectable fat. Hence, in contrast with albino rats, in which in any given genetic setting the total number of adipocytes cannot be reduced once they are formed (27), the number of adipocytes in selected adipose tissues of sand rats may be modulated in both directions by either pharmacological or dietary means.

The hypoglycemic-hypoinsulinemic effect of MEDICA 16 was evaluated here in sand rats maintained at their normoglycemic-hyperinsulinemic stage (11,12) before the development of the fulminant ketotic-diabetic syndrome (6). In contrast with the previously reported lack of insulin receptors in isolated hepatocytes of sand rats (28), the epididymal insulin receptors could still be detected in the obese hyperinsulinemic animals studied. Furthermore, in line with previously reported observations made in other animal models of obesity (29–31), the peripheral resistance to insulin in untreated sand rats was characterized by a pronounced decrease in their epididymal insulin receptors compared with that of calorie-restricted animals. Note, however, that the peripheral resistance to insulin of the obese untreated sand rats could not be accounted for by reduction in their insulin receptors compared with that of calorie-restricted animals, because the eightfold decrease in the apparent number of insulin receptors observed in the obese animals was counteracted by an eightfold increase in their apparent affinity for insulin (Table 6). Similarly, the increase in the apparent number of insulin receptors in MEDICA 16–treated animals was counteracted by a respective decrease in their apparent affinity for insulin (Table 6). The observed increase in the apparent affinity for insulin in larger fat cells corroborates previous findings in Wistar rat epididymal adipocytes derived from aged animals (32) or by mesh filtration (33) but is, however, in contrast with that previously reported for Sprague-Dawley rat epididymal adipocytes (30). The difference could reflect a species-specific capacity for compensating for the decrease in the number of insulin receptors of larger fat cells by an increase in their affinity for insulin. The inverse relationship between the number of insulin receptors and their affinity for insulin could possibly be effected by the mode of embedding the insulin receptors in the plasma membrane as a function of cell size. Thus, a multimeric state of the insulin receptors resulting in a higher affinity for insulin with a concomitant masking of binding sites could perhaps be favored under conditions of an increase in the surface area of the plasma membrane.

By accounting for the apparent number of insulin receptors and their apparent affinity for insulin, the number of receptors actually occupied by insulin at the respective prevailing plasma insulin concentrations (Table 4) can be calculated to be 2100–2400 receptors per epididymal adipocyte for the untreated, MEDICA 16–treated, and calorie-restricted animals. The increase in glucose tolerance as well as in adipose glycogen synthase acitivity by either calorie restriction or MEDICA 16 treatment of obese sand rats, despite the similar occupancy of insulin-binding sites, may indicate that the diabetic pattern of obese sand rats and its reversion by calorie restriction or MEDICA 16 treatment presumably result from a modulation of postreceptor sites due to adipose reduction mediated by the two intervention modes. The sand rat follows in this respect previously reported animal model systems for obesity (29,31,34,35). Note, however, that the postreceptor effects of MEDICA 16 were still dependent on the availability of insulin, as MEDICA 16 could not replace insulin in streptozocin-induced diabetic albino rats. The relationship between the basal conditions set by dietary or pharmacological means and the action of insulin within the postreceptor domain still remains to be investigated.

The combined effects of MEDICA 16 in sand rats may reflect the pharmacological potential of MEDICA compounds in hyperlipidemic-obesity-diabetic syndromes.

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EXHIBIT F

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Hypocholesterolaemic effect of $\beta\beta'$-methyl-substituted hexadecanedioic acid (MEDICA 16) in the male hamster

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Treatment of cholesterol-fed male hamsters kept on a diet of purina chow with $\beta\beta'$-methyl-substituted hexadecanedioic acid (MEDICA 16) resulted in a progressive hypocholesterolaemic effect, amounting to a 50% decrease in the cholesterol content of all plasma lipoproteins. The decrease in plasma cholesterol could be accounted for by activation of plasma-cholesterol efflux through the liver into the bile mediated by MEDICA 16-induced (*a*) increase of the number of liver LDL receptors, (*b*) activation of liver neutral cholesteryl ester hydrolase with a concomitant inhibition of liver acyl-CoA cholesterol acyltransferase, resulting in shifting of the liver cholesteryl ester/free-cholesterol cycle in the direction of free cholesterol, and (*c*) activation of cholesterol efflux from the liver into the bile. The increase in bile cholesterol output was accompanied by an increase in bile phospholipids but not in bile acids. In contrast with rats, MEDICA 16-treatment of male hamsters did not result in a hypotriacylglycerolaemic effect, inhibition of lipogenesis, nor in a substantial decrease in plasma apolipoprotein C-III content.

INTRODUCTION

Tetramethylhexadecanedioic acid (MEDICA 16) has recently been reported to induce a potent hypolipidaemic effect in the normolipaemic or nephrotic hyperlipaemic rat [1]. The observed hypolipidaemic effect consisted of a decrease in the plasma triacylglycerol and cholesterol content of chylomicrons and very-low-density lipoproteins (VLDL) with a concomitant increase in the relative abundance of high-density-lipoprotein (HDL) cholesterol [1]. The hypolipidaemic effect, with respect to plasma VLDL, could be partially accounted for by inhibition of synthesis of liver long-chain fatty acid and cholesterol as a result of a reversible inhibition of ATP citrate lyase [2] and acetyl-CoA carboxylase [3] together with a non-reversible inhibition of cholesterol synthesis at a step beyond the hydroxymethylglutaryl (HMG)-CoA reductase [4]. The overall production rate of chylomicrons remained, however, unaffected by MEDICA 16 treatment [5]. The hypolipidaemic affect with respect to both types of triacylglycerol-rich lipoprotein could be further accounted for by an increase in their plasma clearance accompanied by a 10-fold decrease in plasma apolipoprotein (apo) C-III [1,5]. The reduction in plasma apo CC-III was proposed to drive premature hepatic uptake of plasma triacylglycerol-rich lipoproteins by de-inhibiting the lipoprotein lipase, hepatic triacylglycerol lipase and receptor-mediated liver uptake of the apo C-III-deficient particles [1,5].

The male hamster may offer a better animal model for examining human plasma lipoprotein profiles, lipoprotein metabolism and liver cholesterol homoeostasis than the rat [6–8]. Thus, in contrast with rats, a substantial fraction of plasma cholesterol is carried, in the hamster, by low-density lipoproteins (LDL) and may be further enriched by cholesterol feeding [9]. Moreover, since the rate of rat liver cholesterol synthesis is exceptionally higher than that of other species [7], cholesterol homoeostasis in the rat liver as a function of endogenous or exogenous cholesterol availability is maintained in the first instance by regulating *de novo* cholesterol synthesis [6], and only under conditions where the adaptive synthetic response is blocked or saturated is liver cholesterol homoeostasis regulated by receptor-mediated cholesterol uptake [8]. In contrast with rat, the capacity for liver cholesterol synthesis in human or male hamster is limited and may even be further limited by cholesterol feeding, thus allowing for liver cholesterol homoeostasis to be mediated by cholesterol influx/efflux rather than *de novo* cholesterol synthesis.

To evaluate the hypolipidaemic potential of MEDICA 16 in an animal model for human lipoproteins, and in light of the above considerations, the effect of MEDICA 16 was studied here in cholesterol-fed male hamsters, where a substantial fraction of plasma cholesterol is carried by LDL and where cholesterol homoeostasis may be expected to be accounted for by liver cholesterol traffic rather than *de novo* cholesterol synthesis.

EXPERIMENTAL

Materials

MEDICA 16 was synthesized as previously described [2]. Triacylglycerol, and total cholesterol were determined using Boehringer kits nos. 701912 and 286691 respectively. [1,2-^{3}H]-Cholesterol (60 Ci/mmol), [1-^{14}C]oleoyl-CoA (60 mCi/mmol) and 3-[*glutaryl*-3-^{14}C]hydroxy-3-methylglutaryl-CoA (60 mCi/mmol) were from NEN. Cholesteryl [1-^{14}C]oleate (60 mCi/mmol) and ^{125}I (15.8 mCi/μg of iodine) were obtained from Amersham International. ^3H_2O was from Rotem Industries, Negev, Israel. Alkaline phosphatase (Cat. no. 5130) and 3α-hydroxysteroid dehydrogenase were from Worthington. All other chemicals were from Sigma Chemical Company.

Animals and diets

Male golden Syrian hamsters of the Hebrew University strain weighing 130–150 g were housed in individual cages under

Abbreviations used: MEDICA 16, $\beta\beta'$-methyl-substituted hexadecanedioic acid; VLDL, very-low-density lipoprotein; HDL, high-density lipoprotein; HMG CoA, hydroxymethylglutaryl-CoA; apo, apolipoprotein; LDL, low-density lipoprotein; CETP, cholesteryl ester transfer protein; ACAT, acyl-CoA cholesterol acyltransferase; NCEH, neutral cholesteryl ester hydrolase; PCAT, phosphatidylcholine cholesterol acyltransferase; PMSF, phenylmethanesulphonyl fluoride.

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conditions of alternating periods (12 h) of light (00:00–12:00) and darkness (12:00–00:00). The animals were maintained on a basic diet consisting of 55% (w/w) carbohydrates, 20% (w/w) protein, 5% (w/w) soya oil, 3.4% (w/w) cellulose, 0.05% (w/w) cholesterol, 10% (w/w) moisture and 6.7% (w/w) salt/vitamin mixture (low-cholesterol group) supplemented with 0.5% (w/w) cholesterol where indicated (high-cholesterol group). Following 7–14 days of adjustment to the diet, cholesterol-fed animals were either treated for 30 days with 0.07% (w/w) MEDICA 16 added to the diet or kept untreated. Food consumption/100 g body wt. for MEDICA 16-treated animals was not significantly different from that of non-treated animals. MEDICA 16 treatment of the high-cholesterol groups for one month resulted in a progressive 10±4% (mean ± S.D.) loss in weight, while non-treated animals maintained their initial weight throughout the treatment period. Animals were killed in the middle of the dark period. All care and treatment of animals was in conformity with the Animal Care Guidelines of the Israeli Academy of Sciences.

Lipoprotein profiles

Blood samples were collected in a solution of 0.1% EDTA by heart puncture under ether anaesthesia. Plasma was centrifuged for 20 min at 102000 *g* in a TST 55.5 rotor and the chylomicrons' free plasma was fractionated into VLDL, LDL and HDL by continuous KBr gradient [10]. Cholesterol and triacylglycerol contents were determined using the respective Boehringer kits. Apolipoproteins were subjected to 11% (w/v) SDS/PAGE and isoelectric focusing as previously described [5] and their content determined by densitometry of stained gels [5].

Liver lipid content

Liver triacylglycerol and phospholipid contents were determined in liver samples extracted in 20 volumes of chloroform/methanol (2:1, v/v). The dried lipid extract was solubilized in warm 0.4% SDS. Triacylglycerol was determined using Boehringer kit no. 701912 and phospholipids were determined according to [11]. Liver cholesterol was determined in liver samples ground with anhydrous sodium sulphate [12] and extracted with chloroform/methanol (2:1, v/v). The dried lipid extract was dissolved in propan-2-ol and free cholesterol and cholesteryl ester species were determined by h.p.l.c. [13] using cholesteryl acetate as internal standard. Cholesteryl ester content was calculated by summing up the contents of the three dominant cholesteryl ester species, namely, cholesteryl palmitate, cholesteryl oleate and cholesteryl linoleate. Liver microsomal cholesterol and cholesteryl oleate content were determined in microsomal samples extracted according to [14]. The dried lipid extract was dissolved in acetonitrile and subjected to h.p.l.c. analysis as described above.

Cholesterol in chylomicrons

Cholesterol incorporation into chylomicrons was evaluated in ether-anaesthetized cholesterol-fed animals injected with Triton 1339 (520 mg/kg body wt) into the jugular vein. The anaesthetized animals were bled from the eye choroid plexus at the time of Triton 1339 administration and by heart puncture 1 h later. Blood samples were allowed to clot, and the sera were overlayed with saline and centrifuged in a TST 55.5 rotor at 102000 *g* for 20 min. The chylomicron fraction was sliced off and the cholesterol content as a function of time was determined using Boehringer kit no. 286691. Cholesterol incorporation into chylomicrons was found, under these conditions, to be linear for at least 90 min.

Plasma cholesteryl ester transfer protein (CETP) activity

[1,2-^{3}H]Cholesteryl ester-labelled LDL was prepared by incubating human plasma with [1,2-^{3}H]cholesterol (specific activity 60 Ci/mmol) for 18 h [15] followed by five washings with human erythrocytes and isolation of the LDL fraction by KBr-gradient centrifugation. The labelled LDL fraction was dialysed against 5 mM Tris/HCl (pH 7.4) containing 0.15 M NaCl and 0.5 mM EDTA. CETP activity was measured by a modification of [16,17]. [1,2-^{3}H]Cholesteryl ester-labelled human LDL (81 μg of cholesterol) was incubated with non-labelled human HDL (81 μg of cholesterol) in 10 mM Tris/HCl (pH 7.4) containing 6% (w/v) albumin (essentially fatty-acid free), 1.2 mM 5,5'-dithiobis(2-nitrobenzoic acid) and in the presence or absence of added hamster lipoprotein-deficient plasma [$d > 1.21$ g/ml (5 mg of protein)] in a total volume of 0.5 ml. After incubation for 3 h at 37 °C, 50 μl of unlabelled serum was added, LDL was precipitated by heparin $MnCl_2$, the HDL-cholesteryl ester was extracted according to [14] and subjected to silicic acid t.l.c. in light petroleum (b.p. 40–60 °C)/diethyl ether/acetic acid (75:25:1, by vol.). The cholesteryl ester spot was cut off the plate and counted in scintillation fluid.

Output of biliary lipids

The cystic duct of phenobarbital-anaesthetized hamsters was ligated, followed by cannulation of the common bile duct with PE-10 polyethylene tubing (Clay Adams) and collection of bile for 1 h. Bile-acid content was measured enzymically according to [18]. Bile phospholipids were determined according to [11]. Bile cholesterol content was determined using Boehringer kit no. 286691.

Synthesis of liver lipids

Liver cholesterogenesis and lipogenesis rates were determined by following the rate of ^3H_2O incorporation into liver cholesterol and long-chain fatty acids. The animals were injected intraperitoneally with 50 mCi of ^3H_2O followed 1 h later by quick perfusion of the liver with cold saline [19]. Liver samples were subjected to alkaline hydrolysis, followed by extraction with light petroleum (b.p. 40–60 °C), extensive washings with an ideal upper-phase, digitonin-precipitation of cholesterol, and light petroleum (b.p. 40–60 °C) extraction of the acidified hydrolysate as previously described [2]. Animals perfused for 1 min following the injection of ^3H_2O served as controls.

Liver phospholipid synthesis was determined by measuring the incorporation of glycerol into liver lipids 10 min after the injection of 150 μCi of [1-(3)-^{3}H]glycerol (specific activity 2.9 Ci/mmol) into the jugular vein [20]. Liver lipids were extracted with chloroform/methanol (2:1, v/v) and phospholipid species were separated by t.l.c. using Kieselgel 60 plates (Merck) eluted with acetone/chloroform/methanol/acetic acid/H_2O (8:6:2:2:1, by vol.). Incorporation of the label under these conditions was found to be linear with time for up to 20 min.

Enzymic assays

Liver microsomal HMG-CoA reductase was measured in alkaline-phosphatase-treated microsomes according to [21]. Liver

microsomal acyl-CoA cholesterol acyltransferase (ACAT) activity was measured according to [22] in unwashed microsomes prepared from liver samples homogenized in 4 vol. of 0.25 M sucrose containing 1 mM EDTA (pH 7.4). Liver cytosolic neutral cholesteryl ester hydrolase (NCEH) activity was measured in the 105000 *g* supernatant prepared from liver samples homogenized in 0.15 M potassium phosphate buffer (pH 7.4) containing 10% (w/v) glycerol and using cholesteryl [1-^{14}C]oleate as substrate in a final volume of 0.3 ml [23]. The activity was measured in the presence of 0.5 mg of cytosolic protein and was found to be linear at this range. Concentrations in the range 4–6 mg of cytosolic protein [24] yielded activities non-linear with protein concentrations. The specific activity of the cholesteryl oleate substrate was corrected for the presence of non labelled cytosolic cholesteryl oleate determined by h.p.l.c. as described above. Activity of liver cholesterol 7-α hydroxylase was measured in washed microsomal preparations according to [25] using endogenous cholesterol as substrate. Plasma phosphatidylcholine cholesterol acyltransferase (PCAT) was measured according to [15].

Ligand blotting of LDL receptors

Liver membranes were prepared by Polytron homogenization for 30 s of 2 g of liver pieces suspended in 10 ml of ice-cold buffer, containing 10 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 1 mM $CaCl_2$ and 1 mM phenylmethanesulphonyl fluoride (PMSF). The homogenate was centrifuged at 500 *g* for 5 min followed by centrifugation of the supernatant, first at 8000 *g* for 15 min and finally at 105000 *g* for 60 min. The membrane pellet was suspended in the homogenization buffer and precipitated again at 105000 *g* for 60 min. The washed pellets were frozen in liquid nitrogen and stored at −70 °C. For ligand blotting the membranes were solubilized by passing the pellet through 19-gauge and 23-gauge needles using a solubilization buffer containing 125 mM Tris/maleate (pH 6.0), 2 mM $CaCl_2$, 0.16 M NaCl, 1% (v/v) Triton X-100, 1 mM PMSF, 0.1 mM leupeptin and 1 μg/ml pepstatin and left on ice for 30 min. Insoluble particles were spun away by centrifugation at 125000 *g* for 45 min and the protein content was measured using Bradford reagent. Solubilized membranes (150 μg) were electrophoresed by 7% (w/v) SDS/PAGE and transferred to nitrocellulose paper as described by Semenkovich et al. [26]. The nitrocellulose paper was incubated for 18 h at 4 °C in a blocking buffer containing 50 mM Tris/HCl (pH 8.0), 90 mM NaCl, 5% (w/v) albumin and 2 mM $CaCl_2$, followed by a 5 h incubation at room temperature in the presence of 5 μg/ml rabbit ^{125}I-labelled β-VLDL (300 c.p.m./ng). Finally, the paper was washed five times with the blocking buffer containing 0.5% albumin at room temperature and subjected to autoradiography. β-VLDL-ligand blotting in the absence of calcium added during pre-incubation, incubation and washings served as control. β-VLDL was prepared by feeding a rabbit 2% (w/w) cholesterol for one week. The animal was starved overnight, blood was collected in 0.1% EDTA solution and the plasma was centrifuged for 23 h at 275000 *g* in a SW 41 rotor at a KBr density of 1.019 g/ml. The washed β-VLDL fraction was iodinated using iodine monochloride [27].

Statistics

The significance of differences was evaluated using the Mann–Whitney U-test.

RESULTS

Plasma lipoproteins

The hypercholesterolaemic effect of cholesterol feeding in hamsters and the extent of the hypocholesterolaemic effect of MEDICA 16 in cholesterol-fed hamsters were evaluated here compared with non-treated hamsters maintained on a low-cholesterol diet. The time-course of cholesterol feeding and the hypocholesterolaemic effect of MEDICA 16 are shown in Figure 1. While plasma cholesterol content in cholesterol-fed, non-treated animals progressively increased throughout the feeding period approaching a steady level of approx. 300 mg/100 ml,

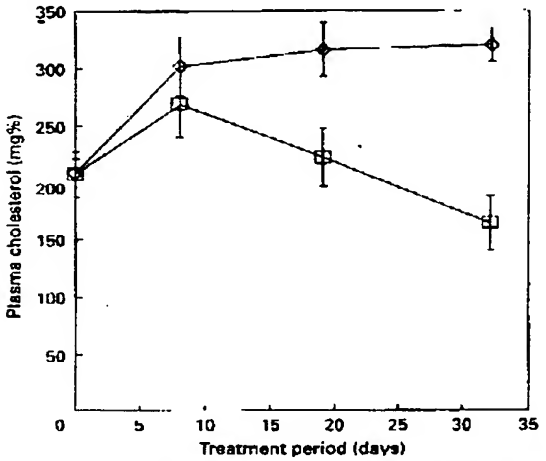

Figure 1 Time course of the hypocholesterolaemic effect of MEDICA 16

Male hamsters were kept on a high cholesterol diet for 47 days. After 14 days of adjustment to cholesterol feeding they were either treated with 0.07% (W/W) MEDICA 16 (□) or kept non-treated (◇) for 33 additional days. Values are given as mean ± S.D. ($n = 6$)

Table 1 Profile of plasma lipoproteins in MEDICA 16-treated hamsters

Male hamsters were kept on low- and high-cholesterol diets in the absence or presence of added 0.07% (w/w) MEDICA 16. After 4 weeks of treatment, the plasma lipoprotein profile was determined as described in the Experimental section. Values are given as means + S.D. ($n = 5$) of one representative experiment out of three. * indicates significantly different from the respective value of the cholesterol-fed non-treated group ($P < 0.05$). † indicates significantly different from the respective low-cholesterol value ($P < 0.05$).

Lipoprotein	Treatment... Low cholesterol diet	High-cholesterol diet	High cholesterol diet, MEDICA 16-treated
Cholesterol (mg/100 ml of plasma)			
Total	132 ± 20	266 ± 29†	141 + 21*
Chylomicrons	4 ± 2	8 ± ?	5 ± 1
VLDL	6 ± 1	18 + 2†	13 ± 2*
LDL	19 ± 2	42 ± 9†	19 ± 8*
HDL	103 ± 12	198 ± 19†	104 + 10*
Triacylglycerol (mg/100 ml of plasma)			
Total	91 ± 23	127 + 37	138 ± 10
Chylomicrons	53 ± 15	57 + 12	57 ± 10
VLDL	24 ± 4	45 ± 4†	55 ± 3†
LDL	13 + 2	18 ± 2	18 ± 3
HDL	1 ± 1	7 ± 4	8 ± 6

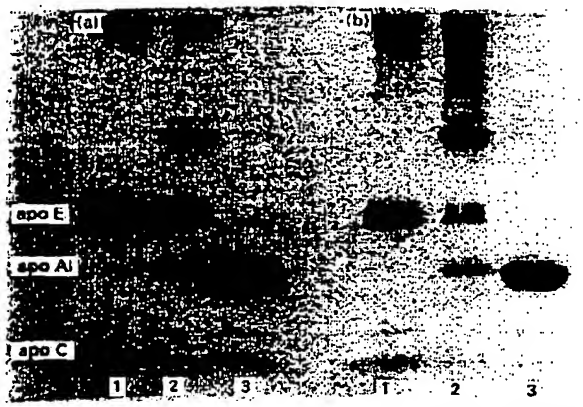

Figure 2 Plasma apolipoproteins profile of cholesterol-fed, MEDICA 16-treated hamsters

Conditions were as described in Table 1. The combined lipoprotein fractions of non-treated (a) and MEDICA 16-treated (b) cholesterol-fed hamsters were subjected to SDS/PAGE as described in the Experimental section. Samples were applied (80 μg of protein/lane) as follows: 1, VLDL; 2, LDL; 3, HDL. The unidentified protein in the LDL fraction is presumably albumin [28].

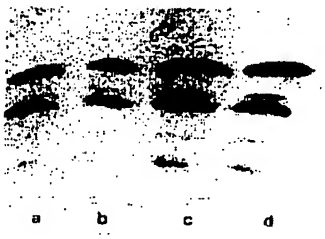

Figure 3 Plasma apo Cs of MEDICA 16-treated hamsters

Conditions were as described in Table 1. The combined HDL (a,b) and VLDL (c,d) fractions of MEDICA 16-treated (a,c) and non-treated (b,d) cholesterol-fed animals were subjected to isoelectric focusing as described in the Experimental section. Samples were applied at 80 μg of protein/lane.

plasma cholesterol content of MEDICA 16-treated cholesterol-fed animals progressively decreased throughout the 1-month-long treatment period, approaching plasma cholesterol levels of animals kept on a low-cholesterol diet (132 ± 20 mg/100 ml).

The lipoprotein profiles induced by cholesterol feeding and MEDICA 16 treatment are shown in Table 1. It is noteworthy that in the low- as well as high-cholesterol groups most of plasma cholesterol is carried by HDL (74–78%) and LDL (14–16%), whereas the contribution made by VLDL- and chylomicron cholesterol was minimal. The overall hypocholesterolaemic effect induced by MEDICA 16 treatment resulted from a 50% decrease in the cholesterol content of all of the lipoprotein fractions. However, most of the hypocholesterolaemic effect could be accounted for by that of HDL cholesterol, this being the most dominant plasma cholesterol fraction.

The apolipoprotein profile induced by MEDICA 16 in cholesterol-fed hamsters is shown in Figures 2 and 3. MEDICA 16 treatment resulted in a significant decrease in the apo E content of VLDL and LDL, while apo AI and apo C levels remained essentially unaffected. The presence of apoE in the LDL fraction is noteworthy. This could either reflect contamination of the LDL fraction by VLDL or the authentic composition of hamster's LDL.

The hypocholesterolaemic effect of MEDICA 16 could not be accounted for by a putative decrease in cholesterol incorporation into chylomicrons. Thus, cholesterol incorporation into chylomicrons measured in cholesterol-fed animals injected with Triton 1339 amounted to 126 ± 11 (mean ± S.D., $n = 9$) and 97 ± 26 ($n = 8$) mg/100 ml of serum per h per kg body wt. in non-treated and MEDICA 16-treated animals respectively (not significantly different at $P = 0.05$).

Plasma PCAT or CETP activities remained unaffected by MEDICA 16 treatment (results not shown).

Liver lipid content

Cholesterol feeding resulted in a > 70-fold increase in liver cholesteryl ester, with only relatively slight changes in liver free cholesterol (Table 2). MEDICA 16 treatment resulted in a pronounced decrease in liver cholesteryl ester while liver free cholesterol content remained essentially unaffected.

Liver microsomal cholesteryl ester and free-cholesterol contents, as a function of cholesterol feeding and MEDICA 16 treatment, reflected total liver cholesterol content as presented above (Table 2). Thus, microsomal cholesteryl oleate content was increased 15-fold by cholesterol feeding and decreased to non-detectable values by MEDICA 16 treatment, while microsomal free cholesterol remained essentially unaffected by either cholesterol feeding or MEDICA 16 treatment.

Liver phospholipid content decreased 20% in cholesterol-fed animals while being reversed back to normal values in MEDICA 16-treated animals.

Liver triacylglycerol levels remained essentially unaffected by either cholesterol feeding or MEDICA 16 treatment.

Table 2 Lipid content of livers of MEDICA 16-treated hamsters

Male hamsters were kept on low- and high-cholesterol diets in the presence or absence of added 0.07% (w/w) MEDICA 16. After 4 weeks of treatment, liver lipids were analysed as described in the Experimental section. Values were given as means ± S.D. Values in parentheses indicate no. of animals used. * indicates significantly different from the respective value of the cholesterol-fed non-treated group ($P < 0.05$). † indicates significantly different from the respective low cholesterol value ($P < 0.05$). Abbreviation: n.d., non-detectable.

Treatment	Liver free cholesterol (mg/g of liver)	Liver cholesteryl ester (mg/g of liver)	Microsomal free cholesterol (μg/mg of protein)	Microsomal cholesteryl oleate (μg/mg of protein)	Liver triacylglycerol (mg/g of liver)	Liver phospholipids (μmol/g of liver)
Low cholesterol diet	1.4 ± 0.3 (3)	n.d. (6)	12.0 ± 5.4 (3)	0.6 ± 0.2 (3)	5.90 ± 1.30 (8)	41.3 ± 2.4 (5)
High-cholesterol diet	3.3 ± 0.9† (6)	68.1 ± 20.0† (6)	13.5 ± 4.0 (3)	8.2 ± 5.2† (3)	4.02 ± 0.89 (3)	32.9 ± 1.7† (3)
High-cholesterol diet, MEDICA 16-treated	2.4 ± 0.5 (6)	10.9 ± 3.5*† (6)	12.7 ± 2.3 (3)	n.d.* (3)	5.00 ± 0.52 (6)	42.5 ± 1.4* (3)

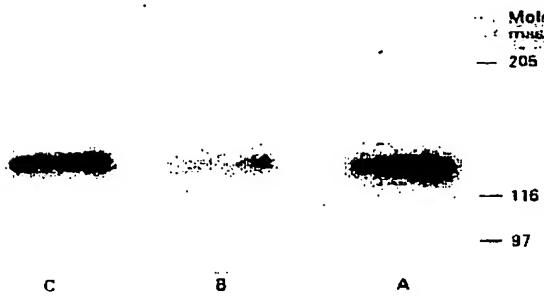

Figure 4 Liver LDL receptors in MEDICA 16-treated hamsters

Conditions were as described in Table 2. LDL receptors were determined by β VLDL blotting as described in the Experimental section. Lane A, high-cholesterol diet, MEDICA 16 treated; lane B, high cholesterol diet only; lane C, low-cholesterol diet only. Results are from one out of three experiments.

Liver activities

Liver LDL-receptor activity, as a function of cholesterol feeding and MEDICA 16 treatment, was evaluated by β-VLDL ligand blotting (Figure 4). Liver LDL-receptor activity was substantially reduced in cholesterol-fed animals [0.67 ± 0.18 relative densitometric units ($n = 3$)] and was increased by MEDICA 16 treatment [1.26 ± 0.42 relative densitometric units ($n = 3$)] to a level similar to that observed in the cholesterol-deficient group [1.0 ± 0.0 relative densitometric units ($n = 3$)].

Liver cytosolic NCEH and microsomal ACAT activities were significantly reduced and increased respectively by cholesterol feeding (Table 3) and were found to be extensively affected by MEDICA 16 treatment of cholesterol-fed animals. Thus, MEDICA 16 was found to inhibit ACAT activity 3.5-fold, while activating NCEH activity 3-fold. The overall effect of MEDICA 16 thus appears to induce liver cholesteryl ester conversion into free cholesterol while inhibiting cholesterol's esterification back into cholesteryl ester, thus antagonizing the cholesterol flux induced by cholesterol feeding.

HMG-CoA reductase was found to respond to the cholesterol status of the liver, being 3-fold suppressed by cholesterol feeding while becoming up-regulated by MEDICA 16 treatment (Table 3). Down-regulation of the HMG CoA reductase activity by cholesterol feeding resulted in a 4-fold inhibition of cholesterol synthesis in cholesterol-fed animals, as deduced from the incorporation of ^3H_2O into liver cholesterol *in vivo* (Table 3). However, up-regulation of the HMG-CoA reductase activity by MEDICA 16 treatment did not result in activation of liver cholesterol synthesis, indicating perhaps that similarly to rats, cholesterol synthesis was inhibited in hamsters by MEDICA 16 at a step beyond the HMG-CoA reductase [4].

Liver phospholipid synthesis was assessed by following the incorporation of radioactive glycerol into liver lipids 10 min after injection of the label into the jugular vein, assuming similar specific activities of hepatic glycerol 3-phosphate following glycerol injection into treated and non-treated animals. Incorporation of the glycerol label into liver phosphatidylcholine was found to be increased 4.3-fold following MEDICA 16 treatment. MEDICA 16 treatment did not result, however, in

Table 3 Liver enzyme activities of MEDICA 16-treated hamsters

Conditions were as described in Table 2. Liver activities were determined as described in the Experimental section. Values are given as means $\pm$ S.D. ($n = 3$) of one representative experiment out of three. * indicates significantly different value from the respective value of the cholesterol-fed non-treated group ($P < 0.05$). † indicates significantly different value from the respective low-cholesterol value ($P < 0.05$).

Treatment	Enzyme activity (pmol/min per mg of protein)				Cholesterol synthesis (nmol of ^3H_2O incorporated/h per g of wet tissue)	Fatty-acid synthesis (nmol of ^3H_2O incorporated/h per g of wet tissue)
	ACAT	NECH	Cholesterol 7 α hydroxylase	HMG-CoA reductase		
Low-cholesterol diet	12.9 ± 3.6	32.1 ± 7.3	-	9.0 ± 2.0	123 ± 40	10914 ± 1915
High-cholesterol diet	44.6 ± 6.3†	15.8 ± 6.0†	4.6 ± 0.2	3.0 ± 0.5†	36 ± 8†	14193 ± 2369
High-cholesterol diet, MEDICA 16-treated	12.8 ± 6.7*	41.5 ± 11.0*	1.6 ± 0.6*	8.2 ± 1.5*	34 ± 5†	13935 ± 4085

Table 4 Biliary-lipid output and content of MEDICA 16-treated hamsters

Male hamsters were kept on a high-cholesterol diet in the absence or presence of added 0.07% MEDICA 16. After 4 weeks of treatment, the bile secretion rate and bile-lipid content were determined as described in the Experimental section. Values are given as mean $\pm$ S.D. ($n = 7$). * indicates value is significantly different from the respective non-treated value ($P < 0.05$). Results are from one representative experiment out of two.

	Bile lipids (μmol/ml)			Bile secretion (ml/kg body wt. per h)	Biliary lipid output (μmol/kg body wt. per h)			Cholesterol mole fraction (%)
	Bile acids	Phospholipids	Cholesterol		Bile acids	Phospholipids	Cholesterol	
Non-treated	12.9 ± 5.6	1.8 ± 0.5	0.18 ± 0.1	1.40 ± 0.73	16.5 ± 7.3	2.3 ± 1.0	0.36 ± 0.16	1.2 ± 0.2
MEDICA 16-treated	7.5 ± 1.6	1.7 ± 0.4	0.35 ± 0.1*	2.97 ± 0.79*	22.0 ± 7.2	5.0 ± 1.7*	0.98 ± 0.37*	3.6 ± 1.1*

changes in the flux of liver lipogenesis, as deduced from the incorporation of ^3H_2O into total liver fatty acids (Table 3).

Biliary lipid output

Biliary lipid output, as a function of MEDICA 16 treatment, is shown in Table 4. MEDICA 16 treatment resulted in 2.7- and 2.1-fold increases in biliary cholesterol and phospholipid output respectively, while bile-acid output was insignificantly increased. The change observed in biliary lipid output was accompanied by a 2.1-fold increase in the bile secretion rate, thus resulting in a decrease in bile-acid concentration but a concomitant 2.4-fold increase in cholesterol (mol fraction).

DISCUSSION

The overall hypocholesterolaemic effect of MEDICA 16 in cholesterol-fed hamsters consists of increasing the cholesterol flux from the plasma compartment into the liver and from the liver into the bile. Since the steady-state levels of free and esterified cholesterol, both in plasma and liver, were reduced by MEDICA 16 treatment, the increased plasma to liver and liver to bile cholesterol fluxes are not accounted for by cholesterol mass action, but presumably reflect MEDICA 16-induced activation of steps controlling the influx and efflux of cholesterol into and out of the liver.

The decrease in plasma cholesterol may indeed be ascribed to a MEDICA 16-induced increase in liver apo B,E receptor activity (Figure 4), resulting in an increased hepatic uptake of apo B,E-containing plasma lipoproteins. The hypocholesterolaemic effect with respect to HDL cholesterol could then result from a concomitant transfer of HDL cholesteryl ester into VLDL and LDL catalysed by CETP present in hamster plasma [16,29]. Alternatively, the decrease in plasma HDL cholesteryl ester could reflect a direct increase in the hepatic uptake of HDL cholesterol mediated by putative HDL receptors or catalysed perhaps by hepatic lipase. The role played by the direct and indirect effects of MEDICA 16 on plasma HDL cholesterol is now being investigated in rats which lack cholesteryl ester transfer activity in their plasma.

The putative increase in plasma cholesterol influx into the liver induced in cholesterol-fed hamsters by MEDICA 16 treatment did not result in flooding of the liver with cholesteryl esters. In fact, liver cholesteryl ester content was found to be dramatically reduced in MEDICA 16-treated animals. The decrease in liver cholesteryl ester may be ascribed to activation of NCEH together with inhibition of liver ACAT activity, resulting in shifting of the cholesteryl ester/free-cholesterol cycle towards free cholesterol. Moreover, MEDICA 16 treatment was found to induce a 3-fold increase in biliary cholesterol output (Table 4), thus pointing to activation of free-cholesterol efflux from the liver. This increase in biliary cholesterol output could not be accounted for by a respective increase in bile-acid output with a concomitant increase in bile-acid-induced cholesterol extraction. MEDICA 16-induced increase of biliary cholesterol output was, however, accompanied by an induced increase in liver phospholipid content, as well as in bile phospholipid output, indicating that most of the cholesterol efflux into bile which was induced by MEDICA 16 treatment was perhaps mediated by vesicular cholesterol transport [30]. Three essential steps are thus proposed to mediate the overall hypocholesterolaemic effect of MEDICA 16 in cholesterol-fed hamsters: an increase in liver LDL-receptor activities resulting in plasma cholesterol influx into the liver, pulling the liver cholesteryl ester/free-cholesterol cycle towards free cholesterol as a result of inhibition and activation of liver ACAT and NCEH respectively, and finally an increase in biliary cholesterol output mediated presumably by vesicular cholesterol transport.

The effect exerted by MEDICA 16 on the various steps involved in plasma and liver cholesterol metabolism in the male hamster may be dissected into steps primarily affected by the drug, and others which presumably respond secondarily to the induced decrease in liver cholesterol. Activation of biliary cholesterol output by MEDICA 16 under conditions where the steady-state level of liver free-cholesterol remained unaffected (Table 2), may indeed be considered as a primary effect of the drug and, as pointed out above, could have resulted from an increase in liver phospholipid synthesis induced by MEDICA 16. MEDICA 16 treatment was reported previously to induce in rats an absolute increase in fatty-acid esterification into liver phospholipids compared with that esterified into liver triacylglycerol [31]. Some other steps, involved in plasma and liver cholesterol metabolism and affected by MEDICA 16, may be considered as responding to the liver cholesterol status rather than being primarily affected by the drug. Liver HMG-CoA reductase, LDL receptors and NCEH were indeed observed to be decreased in this system by cholesterol feeding as previously reported [9,21,24] and increased by MEDICA 16 treatment, thus responding to the liver cholesterol status. Similarly, plasma apo E and liver ACAT were found to be decreased by MEDICA 16, in line with previous reports indicating that these activities are positively correlated with liver cholesterol content [22,32]. It should be pointed out, however, that assuming secondary effects reflecting liver cholesterol status does not rule out a possible primary effect of MEDICA 16 on any of the various liver activities found to be affected by the drug.

The hypolipidaemic effect induced by MEDICA 16 in cholesterol-fed hamsters is different from that previously reported in normo- or hyper-lipaemic rats, both with respect to the phenomenology observed as well as the respective underlying modes of action. Some of the differences are worth noting. The hypolipidaemic effect in normo- or hyper-lipaemic rats consisted of a decrease in plasma cholesterol as well as plasma triacylglycerol [1], while MEDICA 16 treatment of cholesterol-fed hamsters did not result in a triacylglycerolaemic effect. The inefficacy of MEDICA 16 as a hypotriacylglycerolaemic agent in cholesterol-fed hamsters could not be accounted for by cholesterol feeding since it could still be observed in cholesterol-fed rats but not in hamsters maintained on a low-cholesterol diet (N. Mayorek, unpublished). It may be accounted for, however, by the inefficacy of the drug as an inhibitor of the lipogenic pathway (Table 3), or as an effector of plasma apo C-III content (Figures 2 and 3) in hamsters. Finally, it is worth noting that the hypocholesterolaemic effect of MEDICA 16, with respect to HDL cholesterol in hamsters (Table 1), may be related to transfer of cholesteryl ester from HDL to apo B-containing lipoproteins catalysed by CETP, an activity that is lacking in the rat. The differences between rats and hamsters with respect to the hypolipidaemic effect exerted by MEDICA 16 may point to the importance of species-specific factors in defining the overall effect of an hypolipidaemic drug.

MEDICA 16 has been reported previously to act as an adipose-reducing agent in lean rats [33] as well as in animal models for obesity and obesity-induced diabetes [31,34]. The adipose-reductive effect of MEDICA 16 was accounted for by activation of lipolysis in adipose tissue accompanied by increased oxygen consumption [33]. The progressive 10% decrease in weight induced by MEDICA 16 in hamsters after 1 month of treatment may indicate that MEDICA 16 may act as an anti-obesity agent in hamsters as well. In a similar way to adipose reduction in rats,

that observed in cholesterol-fed hamsters was not accounted for by an anorectic effect of the drug.

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EXHIBIT G

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FOREWORD

With this edition, *The Merck Manual* celebrates its 100th birthday. When the editors of the 1st Edition produced their 192-page compendium, they could not have realized the extent to which medical knowledge would explode over the next century. *The Merck Manual* now fills 2,655 pages and covers countless diseases that were not known 100 years ago. A brief review of medical practice as reflected in *The Merck Manual* during the past century follows on page vii.

Although the knowledge of medicine has grown, the goal of *The Merck Manual* has not changed—To provide useful clinical information to practicing physicians, medical students, interns, residents, nurses, pharmacists, and other health care professionals in a concise, complete, and accurate manner. *The Merck Manual* continues to cover all the subjects expected in a textbook of internal medicine as well as detailed information on pediatrics, psychiatry, obstetrics, gynecology, dermatology, pharmacology, ophthalmology, otolaryngology, and a number of special subjects. *The Merck Manual* quickly provides information that helps practitioners achieve optimal care. The more specialized the practice of medicine becomes, the more important such information becomes. Specialists as well as generalists must at some time quickly access information about other specialties.

The 17th edition of *The Merck Manual* is the culmination of an arduous but rewarding 7-year enterprise. Every topic has been updated, and many have been completely rewritten. Topics new to this edition include hand disorders, prion diseases, death and dying, probabilities in clinical medicine, multiple chemical sensitivity, chronic fatigue syndrome, rehabilitation, smoking cessation, and drug therapy in the elderly, among others. The members of the Editorial Board, special consultants, and contributing authors are listed on the following pages with their affiliations. They deserve a degree of gratitude that cannot be adequately expressed here, but we know they will feel sufficiently rewarded if their efforts serve your needs.

Because of the extensive subject matter covered and a successful tradition developed through trials of successes and failures, *The Merck Manual* has some unique characteristics. We urge readers to spend a few minutes reviewing the Guide for Readers (p. xii), the Table of Contents at the beginning of each section (indicated by a thumb tab), and the Index (p. 2657). Subject headings within each section, internal headings within a subject discussion, and boldfaced terms in the text form an outline intended to help with use of the text.

We hope this edition of *The Merck Manual* will serve as an aid to you, our readers, compatible with your needs and worthy of frequent use. Suggestions for improvements will be warmly welcomed and carefully considered.

MARK H. BEERS, M.D., and ROBERT BERKOW, M.D., *Editors*

Test	Specimen	Method	Normal Range: Conventional Units	Normal Range: SI Units
Bilirubin				
Direct	Serum	Colorimetry	≤ 0.4 mg/dl	≤ 7 µmol/L
Indirect	Serum	Colorimetry Calculation: Total bilirubin minus direct bilirubin	≤ 1.0 mg/dL	≤ 22 µmol/L
Total	Serum	Colorimetry	≤ 1.3 mg/dL	≤ 22 µmol/L
Bleeding time, template	Not applicable	Template method	2.5–9.5 min	
CA 15-3	Serum	ABBOTT AXSYM CA 15-3 MEIA	< 32 U/mL	< 32 kU/L
CA 19-9*	Serum	CIS ELSA-CA 19-9 IRMA	< 33 U/mL	< 33 kU/L
CA 27.29	Serum	BIOMIRA TRUQUANT BR RIA	< 38 U/mL	< 38 kU/L
CA 125	Serum	CENTOCOR CA 125 II IRA	< 35 U/mL	< 35 kU/L
Cadmium	Blood	Atomic spectroscopy	< 3 µg/L (nonsmoker)	≤ 17.8 nmol/L (nonsmoker)
Calcitonin	Serum	Immunoassay	Male: < 13.8 pg/mL Female: < 6.4 pg/mL	Male: < 13.8 ng/L Female: < 6.4 ng/L
Calcium	Serum Urine	Colorimetry Colorimetry	8.5–10.3 mg/dL Male: < 300 mg/day Female: < 250 mg/day	2.12–2.57 mmol/L Male: < 7.5 mmol/day Female: < 6.2 mmol/day
Carbon dioxide	Serum	Colorimetry	20–32 mmol/L	20–32 mmol/L
Carboxyhemoglobin	Blood	Spectrophotometry	< 2% of total Hb (nonsmoker)	< 0.02
Carcinoembryonic antigen (CEA)	Serum	CHIRON ACS:180 ICMA	< 2.5 ng/mL (nonsmoker)	< 2.5 µg/L (nonsmoker)
Carotene	Serum	Colorimetry	50–300 µg/dL	0.9–5.6 µmol/L
				[illegible]
Catecholamines Fractionated	Plasma	HPLC	Dopamine Supine: < 90 pg/mL Standing: < 90 pg/mL Epinephrine Supine: < 50 pg/mL Standing: < 90 pg/mL Norepinephrine Supine: 110–410 pg/mL Standing: 125–700 pg/mL	Dopamine Supine: < 589 pmol/L Standing: < 589 pmol/L Epinephrine Supine: < 273 pmol/L Standing: < 491 pmol/L Norepinephrine Supine: 650–2423 pmol/L Standing: 738–4137 pmol/L
Total	Plasma	HPLC	Supine: 120–460 pg/mL Standing: 150–750 pg/mL	Supine: 709–2660 pmol/L Standing: 887–4438 pmol/L
Ceruloplasmin	Serum	Nephelometry	25–63 mg/dL	250–630 mg/L
Chloride	Serum	ISE	95–108 mmol/L	95–108 mmol/L
Cholesterol, total	Serum	Colorimetry	Desirable: < 200 mg/dL Borderline-high: 200–239 mg/dL High: ≥ 240 mg/dL	Desirable: < 5.17 mmol/L Borderline-high: 5.17–6.18 mmol/L High: ≥ 6.21 mmol/L
Complement				
C3	Serum	Nephelometry	70–101 mg/dL	0.78–1.01 g/L
C4	Serum	Nephelometry	10–47 mg/dL	0.16–0.47 g/L
Total (CH_{50})	Serum	Liposome lysis	31–60 U/mL	31–60 kU/L
Complete blood count (CBC)	Blood	Automated hematology analyzer		
Hemoglobin (Hb)			Male: 13.6–17.2 g/dL Female: 12.0–15.6 g/dL	Male: 136–172 g/L Female: 120–156 g/L
Hematocrit (Hct)			Male: 41–50% Female: 35–46%	Male: 0.41–0.50 Female: 0.35–0.46
RBC count			Male: $4.1–5.8 \times 10^6/\mu L$ Female: $3.9–5.2 \times 10^6/\mu L$	Male: $4.1–5.8 \times 10^{12}/L$ Female: $3.9–5.2 \times 10^{12}/L$
RBC indices			Mean corpuscular volume: 78–102 fL Mean corpuscular Hb: 27–33 pg Mean corpuscular Hb concentration: 32–36 g/dL RBC distribution width: ≤ 15%	Mean corpuscular volume: 78–102 fL Mean corpuscular Hb: 27–33 pg Mean corpuscular Hb concentration: 320–360 g/L RBC distribution width: ≤ 0.15
WBC count			$3.8–10.8 \times 10^3/\mu L$	$3.8–10.8 \times 10^9/L$
WBC differential			Absolute neutrophils: 1600–7800 cells/µL Absolute eosinophils: 50–550 cells/µL	Absolute neutrophils: $1.6–7.8 \times 10^9/L$ Absolute eosinophils: $0.05–0.55 \times 10^9/L$

Table continues on the following page.

TABLE 296–2. NORMAL LABORATORY VALUES *(Continued)*

Test	Specimen	Method	Normal Range: Conventional Units	Normal Range: SI Units
Human chorionic gonadotropin (hCG)				
Qualitative	Urine	Immunoassay	Nonpregnant negative Pregnant positive	
Quantitative (intact and free β)	Serum	Immunoassay	Male: < 2 IU/L Female Premenopausal < 5 IU/L Postmenopausal < 10 IU/L Pregnancy 0–2 wk < 500 IU/L 2–3 wk 100–6,000 IU/L 3–4 wk 500–10,000 IU/L 1–2 mo 1,000–200,000 IU/L 2–3 mo 10,000–100,000 IU/L	Male: < 2 IU/L Female Premenopausal < 5 IU/L Postmenopausal < 10 IU/L Pregnancy 0–2 wk < 500 IU/L 2–3 wk 100–6,000 IU/L 3–4 wk 500–10,000 IU/L 1–2 mo 1,000–200,000 IU/L 2–3 mo 10,000–100,000 IU/L
17-Hydroxycorticosteroids	Urine	Enzymatic colorimetry	Male: 3–15 mg/day Female: 2–12 mg/day	Male: 8.3–41.4 μmol/day Female: 5.5–33.1 μmol/day
5-Hydroxyindoleacetic acid (5-HIAA)	Urine	HPLC	0.5–9.0 mg/day	3–47 μmol/day
Immunoglobulin				
IgA	Serum	Nephelometry	81–463 mg/dL	0.81–4.63 g/L
IgD	Serum	Radial immunodiffusion	≤ 14 mg/dL	≤ 0.14 g/L
IgE	Serum	Immunoassay	< 180 U/mL	< 432 μg/L
IgG, subclasses	Serum	Nephelometry	Subclass IgG 1: 450–900 mg/dL Subclass IgG 2: 180–590 mg/dL Subclass IgG 3: 13–80 mg/dL Subclass IgG 4: 8–100 mg/dL	Subclass IgG 1: 4.5–9.0 g/L Subclass IgG 2: 1.8–5.9 g/L Subclass IgG 3: 0.13–0.80 g/L Subclass IgG 4: 0.08–1.00 g/L
IgG, total	Serum	Nephelometry	723–1695 mg/dL	7.23–16.95 g/L
IgM	Serum	Nephelometry	48–271 mg/dL	0.48–2.71 g/L
Insulin	Serum	Immunoassay	6–25 μU/mL	35–179 pmol/L
Iron	Serum	Colorimetry	26–170 μg/dL	4.6–30.4 μmol/L
Iron-binding capacity	Serum	Colorimetry Calculation: % transferrin saturation = (100 × total iron)/total iron-binding capacity	200–450 μg/dL % Saturation: 12–57%	35.8–80.6 μmol/L % Saturation: 0.15–0.57
17-Ketogenic steroids	Urine	Colorimetry	Male: 5–23 mg/day Female: 3–15 mg/day	Male: 17–80 μmol/day Female: 10–52 μmol/day
17-Ketosteroids, total	Urine	Colorimetry	Male: 8–32 mg/day Female: 5–15 mg/day	Male: 30–76 μmol/day Female: 17–52 μmol/day
Lactate dehydrogenase (LD)				
Isoenzymes	Serum	Electrophoresis	LD1: 20–36% of total LD2: 32–50% of total LD3: 18–30% of total LD4: 2–10% of total LD5: 0–18% of total	LD1: 0.20–0.36 of total LD2: 0.32–0.50 of total LD3: 0.18–0.25 of total LD4: 0.02–0.10 of total LD5: 0.00–0.18 of total
Total	Serum	Enzymatic colorimetry	≤ 270 IU/L	≤ 4.5 μkat/L
Lactic acid	Plasma (venous)	Enzymatic colorimetry	9–16 mg/dL	1.0–1.8 mmol/L
Lead	Blood	Atomic spectroscopy	< 25 μg/dL	< 1.21 μmol/L
Lipase	Serum	Enzymatic colorimetry	7–60 U/L	0.12–1.00 μkat/L
Low density lipoprotein (LDL) cholesterol, direct	Serum	Immunoseparation/colorimetry	Desirable: < 130 mg/dL Borderline High 130–159 mg/dL High ≥ 160 mg/dL	Desirable: < 3.36 mmol/L Borderline-high: 3.36–4.11 mmol/L High: ≥ 4.14 mmol/L
Luteinizing hormone (LH)	Serum	Immunoassay	Male: 20–70 yr 1.8–12.9 IU/L ≥ 70 yr 11.3–56.4 IU/L Female: Follicular phase 0.8–25.8 IU/L Midcycle 20.0–87.3 IU/L Luteal phase 0.8–27.1 IU/L Pregnancy < 1.4 IU/L Postmenopausal 5.0–52.3 IU/L	Male: 20–70 yr 1.8–12.9 IU/L ≥ 70 yr 11.3–56.4 IU/L Female: Follicular phase 0.8–25.8 IU/L Midcycle 20.0–87.3 IU/L Luteal phase 0.8–27.1 IU/L Pregnancy < 1.4 IU/L Postmenopausal 5.0–52.3 IU/L

Table continues on the following page.

לשר האוצר שלום

9 עמודים אלה עמודים שלי.

ד. בן-גוריון

7.8.58

EXHIBIT H

Applicants: Jacob Bar-Tana
Serial No.: 10/735,439
Filed: December 11, 2003

Exhibit H

Metabolic syndrome.

1

Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III)

Executive Summary

Applicants: Jacob Bar-Tana
U.S. Serial No.: 10/735,439
Filed: December 11, 2003
Exhibit H

Discrimination Prohibited: Under provisions of applicable public laws enacted by Congress since 1964, no person in the United States shall, on the grounds of race, color, national origin, handicap, or age, be excluded from participation in, be denied the benefits of, or be subjected to discrimination under any program or activity for (on the basis of sex with respect to any education program or activity) receiving Federal financial assistance. In addition, Executive Order 11141 prohibits discrimination on the basis of age by contractors and subcontractors in the performance of Federal contracts, and Executive Order 11246 states that no federally funded contractor may discriminate against any employee or applicant for employment because of race, color, religion, sex, or national origin. Therefore, the National Heart, Lung, and Blood Institute must be operated in compliance with these laws and Executive Orders.

High Blood Cholesterol

Detection

Evaluation

Treatment

Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on

Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III)

Executive Summary

National Cholesterol Education Program
National Heart, Lung, and Blood Institute
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Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III)

Executive Summary

Introduction

The Third Report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III, or ATP III) constitutes the National Cholesterol Education Program's (NCEP's) updated clinical guidelines for cholesterol testing and management. The full ATP III document is an evidence-based and extensively referenced report that provides the scientific rationale for the recommendations contained in the executive summary. ATP III builds on previous ATP reports and expands the indications for intensive cholesterol-lowering therapy in clinical practice. It should be noted that these guidelines are intended to inform, not replace, the physician's clinical judgment, which must ultimately determine the appropriate treatment for each individual.

Background

The third ATP report updates the existing recommendations for clinical management of high blood cholesterol. The NCEP periodically produces ATP clinical updates as warranted by advances in the science of cholesterol management. Each of the guideline reports—ATP I, II, and III—has a major thrust. ATP I outlined a strategy for primary prevention of coronary heart disease (CHD) in persons with high levels of low density lipoprotein (LDL) cholesterol (≥160 mg/dL) or those with borderline-high LDL cholesterol (130-159 mg/dL) and multiple (2+) risk factors. ATP II affirmed the importance of this approach and added a new feature: the intensive management of LDL cholesterol in persons with established CHD. For CHD patients, ATP II set a new, lower LDL cholesterol goal of ≤100 mg/dL. ATP III adds a call for more intensive LDL-lowering therapy in certain groups of people, in accord with recent clinical trial evidence, but its core is based on ATP I and ATP II. Some of the important features shared with previous reports are shown in Table A in the Appendix.

While ATP III maintains attention to intensive treatment of patients with CHD, its major new feature is a focus on primary prevention in persons with multiple risk factors. Many of these persons have a relatively high risk for CHD and will benefit from more intensive LDL-lowering treatment than recommended in ATP II. Table 1 shows the new features of ATP III.

Table 1. New Features of ATP III

Focus on Multiple Risk Factors

- Raises persons with diabetes without CHD, most of whom display multiple risk factors, to the risk level of CHD risk equivalent.
- Uses Framingham projections of 10-year absolute CHD risk (i.e., the percent probability of having a CHD event in 10 years) to identify certain patients with multiple (2+) risk factors for more intensive treatment.
- Identifies persons with multiple metabolic risk factors (metabolic syndrome) as candidates for intensified therapeutic lifestyle changes.

Modifications of Lipid and Lipoprotein Classification

- Identifies LDL cholesterol <100 mg/dL as optimal.
- Raises categorical low HDL cholesterol from <35 mg/dL to <40 mg/dL because the latter is a better measure of a depressed HDL.
- Lowers the triglyceride classification cutpoints to give more attention to moderate elevations.

Support for Implementation

- Recommends a complete lipoprotein profile (total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides) as the preferred initial test, rather than screening for total cholesterol and HDL alone.
- Encourages use of plant stanols/sterols and viscous (soluble) fiber as therapeutic dietary options to enhance lowering of LDL cholesterol.
- Presents strategies for promoting adherence to therapeutic lifestyle changes and drug therapies.
- Recommends treatment beyond LDL lowering for persons with triglycerides ≥200 mg/dL.

LDL Cholesterol: The Primary Target of Therapy

Research from experimental animals, laboratory investigations, epidemiology, and genetic forms of hypercholesterolemia indicate that elevated LDL cholesterol is a major cause of CHD. In addition, recent clinical trials robustly show that LDL-lowering therapy reduces risk for CHD. For these reasons, ATP III continues to identify elevated LDL cholesterol as the primary target of cholesterol-lowering therapy. As a result, the primary goals of therapy and the cutpoints for initiating treatment are stated in terms of LDL.

Risk Assessment: First Step in Risk Management

A basic principle of prevention is that the intensity of risk-reduction therapy should be adjusted to a person's absolute risk. Hence, the first step in selection of LDL-lowering therapy is to assess a person's risk status. Risk assessment requires measurement of LDL cholesterol as part of lipoprotein analysis and identification of accompanying risk determinants.

In all adults aged 20 years or older, a fasting lipoprotein profile (total cholesterol, LDL cholesterol, high density lipoprotein (HDL) cholesterol, and triglyceride) should be obtained once every 5 years. If the testing opportunity is nonfasting, only the values for total cholesterol and HDL cholesterol will be usable. In such a case, if total cholesterol is ≥200 mg/dL or HDL is <40 mg/dL, a followup lipoprotein profile is needed for appropriate management based on LDL. The relationship between LDL cholesterol levels and CHD risk is continuous over a broad range of LDL levels from low to high. Therefore, ATP III adopts the classification of LDL cholesterol levels shown in Table 2, which also shows the classification of total and HDL cholesterol levels.

Table 2. ATP III Classification of LDL, Total, and HDL Cholesterol (mg/dL)

LDL Cholesterol	
<100	Optimal
100-129	Near optimal/above optimal
130-159	Borderline high
160-189	High
≥190	Very high
Total Cholesterol	
<200	Desirable
200-239	Borderline high
≥240	High
HDL Cholesterol	
<40	Low
≥60	High

Risk determinants in addition to LDL-cholesterol include the presence or absence of CHD, other clinical forms of atherosclerotic disease, and the major risk factors other than LDL (see Table 3). (LDL is not counted among the risk factors in Table 3 because the purpose of counting those risk factors is to modify the treatment of LDL.) Based on these other risk determinants, ATP III identifies three categories of risk that modify the goals and modalities of LDL-lowering therapy. Table 4 defines these categories and shows corresponding LDL-cholesterol goals.

Table 3. Major Risk Factors (Exclusive of LDL Cholesterol) That Modify LDL Goals*

- Cigarette smoking
- Hypertension (BP ≥140/90 mmHg or on antihypertensive medication)
- Low HDL cholesterol (<40 mg/dL)†
- Family history of premature CHD (CHD in male first degree relative <55 years; CHD in female first degree relative <65 years)
- Age (men ≥45 years; women ≥55 years)*

* *In ATP III, diabetes is regarded as a CHD risk equivalent.*

† *HDL cholesterol ≥60 mg/dL counts as a "negative" risk factor; its presence removes one risk factor from the total count.*

Table 4. Three Categories of Risk that Modify LDL Cholesterol Goals

Risk Category	LDL Goal (mg/dL)
CHD and CHD risk equivalents	<100
Multiple (2+) risk factors*	<130
Zero to one risk factor	<160

** Risk factors that modify the LDL goal are listed in Table 3*

The category of highest risk consists of CHD and CHD risk equivalents. The latter carry a risk for major coronary events equal to that of established CHD, i.e., >20% per 10 years (i.e., more than 20 of 100 such individuals will develop CHD or have a recurrent CHD event within 10 years). CHD risk equivalents comprise:

- Other clinical forms of atherosclerotic disease (peripheral arterial disease, abdominal aortic aneurysm, and symptomatic carotid artery disease);
- Diabetes;
- Multiple risk factors that confer a 10-year risk for CHD >20%.

Diabetes counts as a CHD risk equivalent because it confers a high risk of new CHD within 10 years, in part because of its frequent association with multiple risk factors. Furthermore, because persons with diabetes who experience a myocardial infarction have an unusually high death rate either immediately or in the long term, a more intensive prevention strategy is warranted. Persons with CHD or CHD risk equivalents have the lowest LDL cholesterol goal (<100 mg/dL).

The second category consists of persons with multiple (2+) risk factors in whom 10-year risk for CHD is ≤20%. Risk is estimated from Framingham risk scores (see Appendix). The major risk factors, exclusive of elevated LDL cholesterol, are used to define the presence of multiple risk factors that modify the goals and cutpoints for LDL-lowering treatment, and these are listed in Table 3. The LDL cholesterol goal for persons with multiple (2+) risk factors is <130 mg/dL.

The third category consists of persons having 0-1 risk factor; with few exceptions, persons in this category have a 10-year risk <10%. Their LDL cholesterol goal is <160 mg/dL.

Method of risk assessment: counting major risk factors and estimating 10-year CHD risk

Risk status in persons *without* clinically manifest CHD or other clinical forms of atherosclerotic disease is determined by a 2-step procedure.

First, the number of risk factors is counted (Table 3). Second, for persons with multiple (2+) risk factors, 10-year risk assessment is carried out with Framingham scoring (see Appendix) to identify individuals whose short-term (10-year) risk warrants consideration of intensive treatment. Estimation of the 10-year CHD risk adds a step to risk assessment beyond risk factor counting, but this step is warranted because it allows better targeting of intensive treatment to people who will benefit from it. When 0-1 risk factor is present, Framingham scoring is not necessary because 10-year risk rarely reaches levels for intensive intervention; a very high LDL level in such a person may nevertheless warrant consideration of drug therapy to reduce long-term risk. Risk factors used in Framingham scoring include age, total cholesterol, HDL cholesterol, blood pressure, and cigarette smoking. Total cholesterol is used for 10-year risk assessment because of a larger and more robust Framingham database for total than for LDL cholesterol, but LDL cholesterol is the primary target of therapy. Framingham scoring divides persons with multiple risk factors into those with 10-year risk for CHD of >20%, 10-20%, and <10%. It should be noted that this 2-step sequence can be reversed with essentially the same results.* Initial risk assessment in ATP III uses the major risk factors to define the core risk status. Only after the core risk status has been determined should any other risk modifiers be taken into consideration for adjusting the therapeutic approach.

Role of other risk factors in risk assessment

ATP III recognizes that risk for CHD is influenced by other factors not included among the major, independent risk factors (Table 3). Among these are *life-habit risk factors* and *emerging risk factors*. The former include obesity, physical inactivity, and atherogenic diet; the latter consist of lipoprotein (a), homocysteine, prothrombotic and proinflammatory factors, impaired fasting glucose, and evidence of subclinical atherosclerotic disease. The *life-habit risk factors* are direct targets for clinical intervention, but are not used to set a lower LDL cholesterol goal of therapy. The *emerging risk factors* do not categorically modify LDL cholesterol goals; however, they appear to contribute to CHD risk to varying degrees and can have utility in selected persons to guide intensity of risk-reduction therapy. Their presence can modulate clinical judgment when making therapeutic decisions.

Metabolic syndrome

Many persons have a constellation of major risk factors, life-habit risk factors, and emerging risk factors that constitute a condition called the

*If Framingham scoring is carried out *before* risk factor counting, persons with <10 percent risk are then divided into those with 2+ risk factors and 0-1 risk factor by risk factor counting to determine the appropriate LDL goal (see Table 4).

metabolic syndrome. Factors characteristic of the metabolic syndrome are abdominal obesity, atherogenic dyslipidemia (elevated triglyceride, small LDL particles, low HDL cholesterol), raised blood pressure, insulin resistance (with or without glucose intolerance), and prothrombotic and proinflammatory states. ATP III recognizes the metabolic syndrome as a secondary target of risk-reduction therapy, after the primary target—LDL cholesterol. Diagnosis and treatment of the metabolic syndrome is described beginning on page 15 under "Benefit Beyond LDL Lowering: The Metabolic Syndrome as a Secondary Target of Therapy."

The link between risk assessment and cost effectiveness

In ATP III, a primary aim is to match intensity of LDL-lowering therapy with absolute risk. Everyone with elevated LDL cholesterol is treated with lifestyle changes that are effective in lowering LDL levels. Persons at relatively high risk are also candidates for drug treatment, which is very effective but entails significant additional expense. The cutpoints for drug treatment are based primarily on risk-benefit considerations: those at higher risk are likely to get greater benefit. However, cutpoints for recommended management based on therapeutic efficacy are checked against currently accepted standards for cost effectiveness. Lifestyle changes are the most cost-effective means to reduce risk for CHD. Even so, to achieve maximal benefit, many persons will require LDL-lowering drugs. Drug therapy is the major expense of LDL-lowering therapy, and it dominates cost-effectiveness analysis. However, the costs of LDL-lowering drugs are currently in flux and appear to be declining. This report recognizes that as drug prices decline it will be possible to extend drug use to lower risk persons and still be cost effective. In addition, ATP III recognizes that some persons with high long-term risk are candidates for LDL-lowering drugs even though use of drugs may not be cost effective by current standards.

Primary Prevention With LDL-Lowering Therapy

Primary prevention of CHD offers the greatest opportunity for reducing the burden of CHD in the United States. The clinical approach to primary prevention is founded on the public health approach that calls for lifestyle changes, including: 1) reduced intakes of saturated fat and cholesterol, 2) increased physical activity, and 3) weight control, to lower population cholesterol levels and reduce CHD risk, but the clinical approach intensifies preventive strategies for higher risk persons. One aim of primary prevention is to reduce long-term risk (>10 years) as well as short-term risk (≤10 years). LDL goals in primary prevention depend on a person's absolute risk for CHD (i.e., the probability of having a CHD

event in the short term or the long term)—the higher the risk, the lower the goal. Therapeutic lifestyle changes are the foundation of clinical primary prevention. Nonetheless, some persons at higher risk because of high or very high LDL cholesterol levels or because of multiple risk factors are candidates for LDL-lowering drugs. Recent primary prevention trials show that LDL-lowering drugs reduce risk for major coronary events and coronary death even in the short term.

Any person with elevated LDL cholesterol or other form of hyperlipidemia should undergo clinical or laboratory assessment to rule out secondary dyslipidemia before initiation of lipid-lowering therapy. Causes of secondary dyslipidemia include:

- Diabetes
- Hypothyroidism
- Obstructive liver disease
- Chronic renal failure
- Drugs that increase LDL cholesterol and decrease HDL cholesterol (progestins, anabolic steroids, and corticosteroids).

Once secondary causes have been excluded or, if appropriate, treated, the goals for LDL-lowering therapy in primary prevention are established according to a person's risk category (Table 4).

Secondary Prevention With LDL-Lowering Therapy

Recent clinical trials demonstrate that LDL-lowering therapy reduces total mortality, coronary mortality, major coronary events, coronary artery procedures, and stroke in persons with established CHD. As shown in Table 2, an LDL cholesterol level of <100 mg/dL is *optimal;* therefore, ATP III specifies an LDL cholesterol <100 mg/dL as the goal of therapy in secondary prevention. This goal is supported by clinical trials with both clinical and angiographic endpoints and by prospective epidemiological studies. The same goal should apply for persons with CHD risk equivalents. When persons are hospitalized for acute coronary syndromes or coronary procedures, lipid measures should be taken on admission or within 24 hours. These values can guide the physician on initiation of LDL-lowering therapy before or at discharge. Adjustment of therapy may be needed after 12 weeks.

LDL-Lowering Therapy in Three Risk Categories

The two major modalities of LDL-lowering therapy are *therapeutic lifestyle changes* (TLC) and *drug therapy.* Both are described in more detail later. The TLC Diet stresses reductions in saturated fat and cholesterol intakes. When the metabolic syndrome or its associated lipid risk factors (elevated

triglyceride or low HDL cholesterol) are present, TLC also stresses weight reduction and increased physical activity. Table 5 defines LDL cholesterol goals and cutpoints for initiation of TLC and for drug consideration for persons with three categories of risk: CHD and CHD risk equivalents; multiple (2+) risk factors (10-year risk 10-20% and <10%); and 0-1 risk factor.

Table 5: LDL Cholesterol Goals and Cutpoints for Therapeutic Lifestyle Changes (TLC) and Drug Therapy in Different Risk Categories.

Risk Category	LDL Goal	LDL Level at Which to Initiate Therapeutic Lifestyle Changes (TLC)	LDL Level at Which to Consider Drug Therapy
CHD or CHD Risk Equivalents (10-year risk >20%)	<100 mg/dL	≥100 mg/dL	≥130 mg/dL (100-129 mg/dL: drug optional)*
2+ Risk Factors (10-year risk ≤20%)	<130 mg/dL	≥130 mg/dL	10-year risk 10-20%: ≥130 mg/dL 10-year risk <10%: ≥160 mg/dL
0-1 Risk Factor†	<160 mg/dL	≥160 mg/dL	≥190 mg/dL (160-189 mg/dL: LDL-lowering drug optional)

* *Some authorities recommend use of LDL-lowering drugs in this category if an LDL cholesterol <100 mg/dL cannot be achieved by therapeutic lifestyle changes. Others prefer use of drugs that primarily modify triglycerides and HDL, e.g., nicotinic acid or fibrate. Clinical judgment also may call for deferring drug therapy in this subcategory.*

† *Almost all people with 0-1 risk factor have a 10-year risk <10%, thus 10-year risk assessment in people with 0-1 risk factor is not necessary.*

CHD and CHD risk equivalents

For persons with CHD and CHD risk equivalents, LDL-lowering therapy greatly reduces risk for major coronary events and stroke and yields highly favorable cost-effectiveness ratios. The cut-points for initiating lifestyle and drug therapies are shown in Table 5.

- *If baseline LDL cholesterol is ≥130 mg/dL,* intensive lifestyle therapy and maximal control of other risk factors should be started. Moreover, for most patients, an LDL-lowering drug will be required to achieve an LDL cholesterol <100 mg/dL; thus an LDL cholesterol lowering drug can be started simultaneously with TLC to attain the goal of therapy.

- *If LDL cholesterol levels are 100-129 mg/dL,* either at baseline or on LDL-lowering therapy, several therapeutic approaches are available:

- Initiate or intensify lifestyle and/or drug therapies specifically to lower LDL.
- Emphasize weight reduction and increased physical activity in persons with the metabolic syndrome.
- Delay use or intensification of LDL-lowering therapies and institute treatment of other lipid or nonlipid risk factors; consider use of other lipid-modifying drugs (e.g., nicotinic acid or fibric acid) if the patient has elevated triglyceride or low HDL cholesterol.

- *If baseline LDL cholesterol is <100 mg/dL,* further LDL-lowering therapy is not required. Patients should nonetheless be advised to follow the TLC Diet on their own to help keep the LDL level optimal. Several clinical trials are currently underway to assess benefit of lowering LDL cholesterol to well below 100 mg/dL. At present, emphasis should be placed on controlling other lipid and nonlipid risk factors and on treatment of the metabolic syndrome, if present.

Multiple (2+) risk factors and 10-year risk ≤20%

For persons with multiple (2+) risk factors and 10-year risk ≤ 20%, intensity of therapy is adjusted according to 10-year risk and LDL cholesterol level. The treatment approach for each category is summarized in Table 5.

- *Multiple (2+) risk factors and a 10-year risk of 10-20%.* In this category, the goal for LDL cholesterol is <130 mg/dL. The therapeutic aim is to reduce short-term risk as well as long-term risk for CHD. If baseline LDL cholesterol is ≥130 mg/dL, TLC is initiated and maintained for 3 months. If LDL remains ≥130 mg/dL after 3 months of TLC, consideration can be given to starting an LDL-lowering drug to achieve the LDL goal of <130 mg/dL. Use of LDL-lowering drugs at this risk level reduces CHD risk and is cost-effective. If the LDL falls to less than 130 mg/dL on TLC alone, TLC can be continued without adding drugs. In older persons (≥65 years), clinical judgment is required for how intensively to apply these guidelines; a variety of factors, including concomitant illnesses, general health status, and social issues may influence treatment decisions and may suggest a more conservative approach.

- *Multiple (2+) risk factors and a 10-year risk of <10%.* In this category, the goal for LDL cholesterol also is <130 mg/dL. The therapeutic aim, however, is primarily to reduce longer-term risk. If baseline LDL cholesterol is ≥130 mg/dL, the TLC Diet is initiated to reduce LDL cholesterol. If LDL is <160 mg/dL on TLC alone, it should be continued. LDL-lowering drugs generally are not recommended because the patient is not at high short-term risk. On the other hand, if

LDL cholesterol is ≥160 mg/dL, drug therapy can be considered to achieve an LDL cholesterol <130 mg/dL; the primary aim is to reduce long-term risk. Cost-effectiveness is marginal, but drug therapy can be justified to slow development of coronary atherosclerosis and to reduce long-term risk for CHD.

Zero to one risk factor

Most persons with 0-1 risk factor have a 10-year risk <10%. They are managed according to Table 5. The goal for LDL cholesterol in this risk category is <160 mg/dL. The primary aim of therapy is to reduce long-term risk. First-line therapy is TLC. If after 3 months of TLC the LDL cholesterol is <160 mg/dL, TLC is continued. However, if LDL cholesterol is 160-189 mg/dL after an adequate trial of TLC, drug therapy is *optional* depending on clinical judgment. Factors favoring use of drugs include:

- A severe single risk factor (heavy cigarette smoking, poorly controlled hypertension, strong family history of premature CHD, or very low HDL cholesterol);
- Multiple life-habit risk factors and emerging risk factors (if measured);
- 10-year risk approaching 10% (if measured; see Appendix).

If LDL cholesterol is ≥190 mg/dL despite TLC, drug therapy should be considered to achieve the LDL goal of <160 mg/dL.

The purpose of using LDL-lowering drugs in persons with 0-1 risk factor and elevated LDL cholesterol (≥160 mg/dL) is to slow the development of coronary atherosclerosis, which will reduce long-term risk. This aim may conflict with cost-effectiveness considerations; thus, clinical judgment is required in selection of persons for drug therapy, although a strong case can be made for using drugs when LDL cholesterol is ≥190 mg/dL after TLC.

For persons whose LDL cholesterol levels are already below goal levels upon first encounter, instructions for appropriate changes in life habits, periodic followup, and control of other risk factors are needed.

Therapeutic Lifestyle Changes in LDL-Lowering Therapy

ATP III recommends a multifaceted lifestyle approach to reduce risk for CHD. This approach is designated *therapeutic lifestyle changes (TLC).* Its essential features are:

- Reduced intakes of saturated fats (<7% of total calories) and cholesterol (<200 mg per day) (see Table 6 for overall composition of the TLC Diet)
- Therapeutic options for enhancing LDL lowering such as plant stanols/sterols (2 g/day) and increased viscous (soluble) fiber (10-25 g/day)
- Weight reduction
- Increased physical activity

Table 6. Nutrient Composition of the TLC Diet

Nutrient	Recommended Intake
Saturated fat*	Less than 7% of total calories
Polyunsaturated fat	Up to 10% of total calories
Monounsaturated fat	Up to 20% of total calories
Total fat	25-35% of total calories
Carbohydrate†	50-60% of total calories
Fiber	20-30 g/day
Protein	Approximately 15% of total calories
Cholesterol	Less than 200 mg/day
Total calories (energy)‡	Balance energy intake and expenditure to maintain desirable body weight/prevent weight gain

* *Trans fatty acids are another LDL-raising fat that should be kept at a low intake.*

† *Carbohydrate should be derived predominantly from foods rich in complex carbohydrates including grains, especially whole grains, fruits, and vegetables.*

‡ *Daily energy expenditure should include at least moderate physical activity (contributing approximately 200 Kcal per day).*

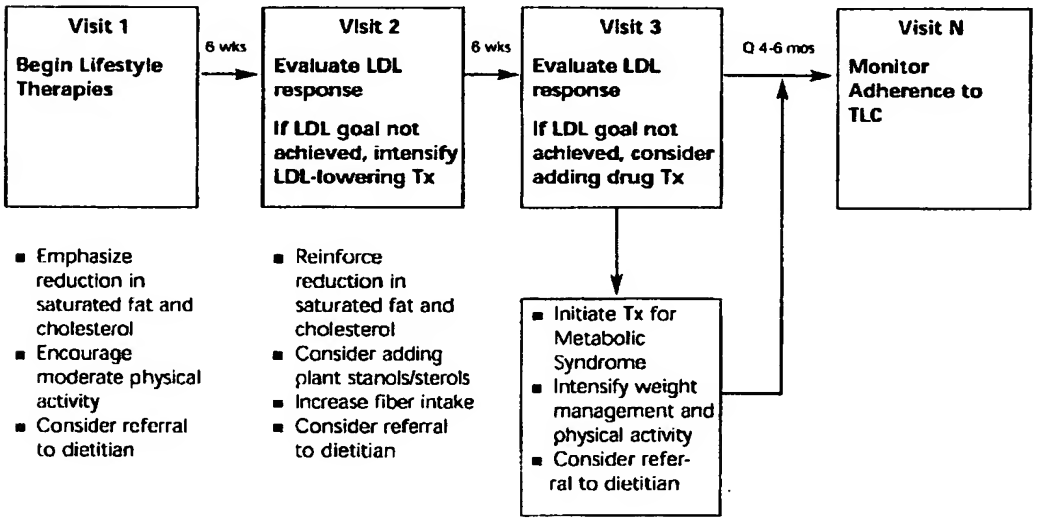
A model of steps in TLC is shown in Figure 1. To initiate TLC, intakes of saturated fats and cholesterol are reduced first to lower LDL cholesterol. To improve overall health, ATP III's TLC Diet generally contains the recommendations embodied in the Dietary Guidelines for Americans 2000. One exception is that total fat is allowed to range from 25-35% of total calories provided saturated fats and *trans* fatty acids are kept low. A higher intake of total fat, mostly in the form of unsaturated fat, can help to reduce triglycerides and raise HDL cholesterol in persons with the metabolic syndrome. In accordance with the Dietary Guidelines, moderate physical activity is encouraged. After 6 weeks, the LDL response is determined; if the LDL cholesterol goal has not been achieved, other therapeutic options for LDL lowering such as plant stanol/sterols and viscous fiber can be added.

After maximum reduction of LDL cholesterol with dietary therapy, emphasis shifts to management of the metabolic syndrome and associated lipid risk factors. The majority of persons with these latter abnormalities are overweight or obese and sedentary. Weight reduction therapy for overweight or obese patients will enhance LDL lowering and will provide other health benefits including modifying other lipid and nonlipid risk factors.

Assistance in the management of overweight and obese persons is provided by the *Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults* from the NHLBI Obesity Education Initiative (1998). Additional risk reduction can be achieved by simultaneously increasing physical activity.

At all stages of dietary therapy, physicians are encouraged to refer patients to registered dietitians or other qualified nutritionists for *medical nutrition therapy*, which is the term for the nutritional intervention and guidance provided by a nutrition professional.

Figure 1. A Model of Steps in Therapeutic Lifestyle Changes (TLC)



Drug Therapy to Achieve LDL Cholesterol Goals

A portion of the population whose short-term or long-term risk for CHD is high will require LDL-lowering drugs in addition to TLC to reach the designated goal for LDL cholesterol (see Table 5). When drugs are prescribed, attention to TLC should always be maintained and reinforced. Currently available drugs that affect lipoprotein metabolism and their major characteristics are listed in Table 7.

Some cholesterol-lowering agents are currently available over-the-counter (OTC) (e.g., nicotinic acid), and manufacturers of several classes of LDL-lowering drugs (e.g., statins, bile acid sequestrants) have applied to the

Table 7. Drugs Affecting Lipoprotein Metabolism

Drug Class, Agents and Daily Doses	Lipid/Lipoprotein Effects		Side Effects	Contraindications	Clinical Trial Results
HMG CoA reductase inhibitors (statins)*	LDL HDL TG	↓18-55% ↑5-15% ↓7-30%	Myopathy Increased liver enzymes	Absolute: • Active or chronic liver disease Relative: • Concomitant use of certain drugs†	Reduced major coronary events, CHD deaths, need for coronary procedures, stroke, and total mortality
Bile acid Sequestrants‡	LDL HDL TG	↓15-30% ↑3-5% No change or increase	Gastrointestinal distress Constipation Decreased absorption of other drugs	Absolute: • dysbetalipoproteinemia • TG >400 mg/dL Relative: • TG >200 mg/dL	Reduced major coronary events and CHD deaths
Nicotinic acid¥	LDL HDL TG	↓ 5-25% ↑15-35% ↓20-50%	Flushing Hyperglycemia Hyperuricemia (or gout) Upper GI distress Hepatotoxicity	Absolute: • Chronic liver disease • Severe gout Relative: • Diabetes • Hyperuricemia • Peptic ulcer disease	Reduced major coronary events, and possibly total mortality
Fibric acids§	LDL *(may be increased in patients with high TG)* HDL TG	↓5-20% ↑10-20% ↓20-50%	Dyspepsia Gallstones Myopathy Unexplained non-CHD deaths in WHO study	Absolute: • Severe renal disease • Severe hepatic disease	Reduced major coronary events

* *Lovastatin (20-80 mg), pravastatin (20-40 mg), simvastatin (20-80 mg), fluvastatin (20-80 mg), atorvastatin (10-80 mg), cerivastatin (0.4-0.8 mg).*

† *Cyclosporine, macrolide antibiotics, various antifungal agents and cytochrome P-450 inhibitors (fibrates and niacin should be used with appropriate caution).*

‡ *Cholestyramine (4-16 g), colestipol (5-20 g), colesevelam (2.6-3.8 g).*

¥ *Immediate release (crystalline) nicotinic acid (1.5-3 g), extended release nicotinic acid (Niaspan ®) (1-2 g), sustained release nicotinic acid (1-2 g).*

§ *Gemfibrozil (600 mg BID), fenofibrate (200 mg), clofibrate (1000 mg BID).*

Food and Drug Administration (FDA) to allow these agents to become OTC medications. At the time of publication of ATP III, the FDA has not granted permission for OTC status for statins or bile acid sequestrants. If an OTC cholesterol-lowering drug is or becomes available, patients should continue to consult with their physicians about whether to initiate drug treatment, about setting the goals of therapy, and about monitoring for therapeutic responses and side effects.

Secondary prevention: drug therapy for CHD and CHD risk equivalents

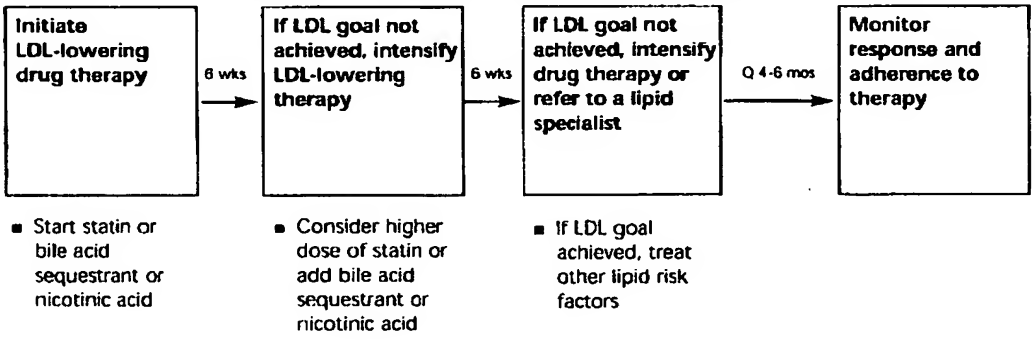
For persons with CHD and CHD risk equivalents, the goal is to attain an LDL cholesterol level <100 mg/dL. The cutpoints for initiating lifestyle and drug therapies are shown in Table 5, and the approach to treatment is discussed immediately after Table 5. Most CHD patients will need LDL-lowering drug therapy. Other lipid risk factors may also warrant consideration of drug treatment. Whether or not lipid-modifying drugs are used, nonlipid risk factors require attention and favorable modification.

In persons admitted to the hospital for a major coronary event, LDL cholesterol should be measured on admission or within 24 hours. This value can be used for treatment decisions. In general, persons hospitalized for a coronary event or procedure should be discharged on drug therapy if the LDL cholesterol is ≥130 mg/dL. If the LDL is 100–129 mg/dL, clinical judgment should be used in deciding whether to initiate drug treatment at discharge, recognizing that LDL cholesterol levels begin to decline in the first few hours after an event and are significantly decreased by 24-48 hours and may remain low for many weeks. Thus, the initial LDL cholesterol level obtained in the hospital may be substantially lower than is usual for the patient. Some authorities hold drug therapy should be initiated whenever a patient hospitalized for a CHD-related illness is found to have an LDL cholesterol >100 mg/dL. Initiation of drug therapy at the time of hospital discharge has two advantages. First, at that time patients are particularly motivated to undertake and adhere to risk-lowering interventions; and second, failure to initiate indicated therapy early is one of the causes of a large "treatment gap," because outpatient followup is often less consistent and more fragmented.

LDL-lowering drug therapy for primary prevention

Table 5 shows the cutpoints for considering drug treatment in primary prevention. The general approach to management of drug therapy for primary prevention is outlined in Figure 2.

Figure 2. Progression of Drug Therapy in Primary Prevention



When drug therapy for primary prevention is a consideration, the third visit of dietary therapy (see Figure 1) will typically be the visit to initiate drug treatment. Even if drug treatment is started, TLC should be continued. As with TLC, the first priority of drug therapy is to achieve the goal for LDL cholesterol. For this reason, an LDL-lowering drug should be started. The usual drug will be a statin, but alternatives are a bile acid sequestrant or nicotinic acid. In most cases, the statin should be started at a moderate dose. In many patients, the LDL cholesterol goal will be achieved, and higher doses will not be necessary. The patient's response should be checked about 6 weeks after starting drug therapy. If the goal of therapy has been achieved, the current dose can be maintained. However, if the goal has not been achieved, LDL-lowering therapy can be intensified, either by increasing the dose of statin or by combining a statin with a bile acid sequestrant or nicotinic acid.

After 12 weeks of drug therapy, the response to therapy should again be assessed. If the LDL cholesterol goal is still not achieved, consideration can be given to further intensification of drug therapy. If the LDL goal cannot be attained by standard lipid-lowering therapy, consideration should be given to seeking consultation from a lipid specialist. Once the goal for LDL cholesterol has been attained, attention can turn to other lipid risk factors and nonlipid factors. Thereafter, patients can be monitored for response to therapy every 4 to 6 months, or more often if considered necessary.

Benefit Beyond LDL Lowering: The Metabolic Syndrome as a Secondary Target of Therapy

Evidence is accumulating that risk for CHD can be reduced beyond LDL-lowering therapy by modification of other risk factors. One potential

secondary target of therapy is the metabolic syndrome, which represents a constellation of lipid and nonlipid risk factors of metabolic origin. This syndrome is closely linked to a generalized metabolic disorder called *insulin resistance* in which the normal actions of insulin are impaired. Excess body fat (particularly abdominal obesity) and physical inactivity promote the development of insulin resistance, but some individuals also are genetically predisposed to insulin resistance.

The risk factors of the metabolic syndrome are highly concordant; in aggregate they enhance risk for CHD at any given LDL cholesterol level. For purposes of ATP III, the diagnosis of the metabolic syndrome is made when three or more of the risk determinants shown in Table 8 are present. These determinants include a combination of categorical and borderline risk factors that can be readily measured in clinical practice.

Table 8. Clinical Identification of the Metabolic Syndrome

Risk Factor	Defining Level
• Abdominal Obesity*	Waist Circumference†
Men	>102 cm (>40 in)
Women	>88 cm (>35 in)
• Triglycerides	≥150 mg/dL
• HDL cholesterol	
Men	<40 mg/dL
Women	<50 mg/dL
• Blood pressure	≥130/≥85 mmHg
Fasting glucose	≥110 mg/dL

* *Overweight and obesity are associated with insulin resistance and the metabolic syndrome. However, the presence of abdominal obesity is more highly correlated with the metabolic risk factors than is an elevated body mass index (BMI). Therefore, the simple measure of waist circumference is recommended to identify the body weight component of the metabolic syndrome.*

† *Some male patients can develop multiple metabolic risk factors when the waist circumference is only marginally increased, e.g., 94-102 cm (37-39 in). Such patients may have a strong genetic contribution to insulin resistance. They should benefit from changes in life habits, similarly to men with categorical increases in waist circumference.*

Management of the metabolic syndrome has a two-fold objective: (1) to reduce underlying causes (i.e., obesity and physical inactivity), and (2) to treat associated nonlipid and lipid risk factors.

Management of underlying causes of the metabolic syndrome

First-line therapies for all lipid and nonlipid risk factors associated with the metabolic syndrome are weight reduction and increased physical activity, which will effectively reduce all of these risk factors. Therefore, after

appropriate control of LDL cholesterol, TLC should stress weight reduction and physical activity if the metabolic syndrome is present.

Weight control. In ATP III overweight and obesity are recognized as major, underlying risk factors for CHD and identified as direct targets of intervention. Weight reduction will enhance LDL lowering and reduce all of the risk factors of the metabolic syndrome. The recommended approaches for reducing overweight and obesity are contained in the clinical guidelines of the NHLBI Obesity Education Initiative.

Physical activity. Physical inactivity is likewise a major, underlying risk factor for CHD. It augments the lipid and nonlipid risk factors of the metabolic syndrome. It further may enhance risk by impairing cardiovascular fitness and coronary blood flow. Regular physical activity reduces very low density lipoprotein (VLDL) levels, raises HDL cholesterol, and in some persons, lowers LDL levels. It also can lower blood pressure, reduce insulin resistance, and favorably influence cardiovascular function. Thus, ATP III recommends that regular physical activity become a routine component in management of high serum cholesterol. The evidence base for this recommendation is contained in the *U.S. Surgeon General's Report on Physical Activity.*

Specific Treatment of Lipid and Non-Lipid Risk Factors

Beyond the underlying risk factors, therapies directed against the lipid and nonlipid risk factors of the metabolic syndrome will reduce CHD risk. These include treatment of hypertension, use of aspirin in patients with CHD to reduce the prothrombotic state (guidelines for aspirin use in primary prevention have not been firmly established), and treatment of elevated triglycerides and low HDL cholesterol as discussed below under Management of Specific Dyslipidemias.

Special Issues

Management of Specific Dyslipidemias

Very high LDL cholesterol (≥190 mg/dL). Persons with very high LDL cholesterol usually have genetic forms of hypercholesterolemia: monogenic familial hypercholesterolemia, familial defective apolipoprotein B, and polygenic hypercholesterolemia. Early detection of these disorders through cholesterol testing in young adults is needed to prevent premature CHD. Family testing is important to identify similarly affected relatives. These

disorders often require combined drug therapy (statin + bile acid sequestrant) to achieve the goals of LDL-lowering therapy.

Elevated serum triglycerides. Recent meta-analyses of prospective studies indicate that elevated triglycerides are also an independent risk factor for CHD. Factors contributing to elevated (higher than normal) triglycerides in the general population include: obesity and overweight, physical inactivity, cigarette smoking, excess alcohol intake, high carbohydrate diets (>60% of energy intake), several diseases (e.g., type 2 diabetes, chronic renal failure, nephrotic syndrome), certain drugs (e.g., corticosteroids, estrogens, retinoids, higher doses of beta-adrenergic blocking agents), and genetic disorders (familial combined hyperlipidemia, familial hypertriglyceridemia, and familial dysbetalipoproteinemia).

In clinical practice, elevated serum triglycerides are most often observed in persons with the metabolic syndrome, although secondary or genetic factors can heighten triglyceride levels. ATP III adopts the following classification of serum triglycerides:

- Normal triglycerides: <150 mg/dL
- Borderline-high triglycerides: 150-199 mg/dL
- High triglycerides: 200-499 mg/dL
- Very high triglycerides: ≥500 mg/dL

The finding that elevated triglycerides are an independent CHD risk factor suggests that some triglyceride-rich lipoproteins are atherogenic. The latter are partially degraded VLDL, commonly called *remnant lipoproteins.* In clinical practice, VLDL cholesterol is the most readily available measure of atherogenic remnant lipoproteins. Thus, VLDL cholesterol can be a target of cholesterol-lowering therapy. ATP III identifies the sum of LDL+VLDL cholesterol [termed *non-HDL cholesterol* (total cholesterol minus HDL cholesterol)] as a secondary target of therapy in persons with high triglycerides (≥200 mg/dL). The goal for non-HDL cholesterol in persons with high serum triglycerides can be set at 30 mg/dL higher than that for LDL cholesterol (Table 9) on the premise that a VLDL cholesterol level ≤30 mg/dL is normal.

The treatment strategy for elevated triglycerides depends on the causes of the elevation and its severity. For all persons with elevated triglycerides, the primary aim of therapy is to achieve the target goal for LDL cholesterol. When triglycerides are *borderline high* (150-199 mg/dL), emphasis should also be placed on weight reduction and increased physical activity. For *high triglycerides* (200-499 mg/dL), non-HDL cholesterol becomes a secondary

Table 9. Comparison of LDL Cholesterol and Non-HDL Cholesterol Goals for Three Risk Categories

Risk Category	LDL Goal (mg/dL)	Non-HDL-C Goal (mg/dL)
CHD and CHD Risk Equivalent (10-year risk for CHD >20%)	<100	<130
Multiple (2+) Risk Factors and 10-year risk ≤20%	<130	<160
0-1 Risk Factor	<160	<190

target of therapy. Aside from weight reduction and increased physical activity, drug therapy can be considered in high-risk persons to achieve the non-HDL cholesterol goal. There are two approaches to drug therapy. First, the non-HDL cholesterol goal can be achieved by intensifying therapy with an LDL-lowering drug; or second, nicotinic acid or fibrate can be added, if used with appropriate caution, to achieve the non-HDL cholesterol goal by further lowering of VLDL cholesterol. In rare cases in which triglycerides are *very high* (≥500 mg/dL), the initial aim of therapy is to prevent acute pancreatitis through triglyceride lowering. This approach requires very low fat diets (≤15% of calorie intake), weight reduction, increased physical activity, and usually a triglyceride-lowering drug (fibrate or nicotinic acid). Only after triglyceride levels have been lowered to <500 mg/dL should attention turn to LDL lowering to reduce risk for CHD.

Low HDL cholesterol. Low HDL cholesterol is a strong independent predictor of CHD. In ATP III, low HDL cholesterol is defined categorically as a level <40 mg/dL, a change from the level of <35 mg/dL in ATP II. In the present guidelines, low HDL cholesterol both modifies the goal for LDL-lowering therapy and is used as a risk factor to estimate 10-year risk for CHD.

Low HDL cholesterol levels have several causes, many of which are associated with insulin resistance, i.e., elevated triglycerides, overweight and obesity, physical inactivity, and type 2 diabetes. Other causes are cigarette smoking, very high carbohydrate intakes (>60% of calories), and certain drugs (e.g., beta-blockers, anabolic steroids, progestational agents)

ATP III does not specify a goal for HDL raising. Although clinical trial results suggest that raising HDL will reduce risk, the evidence is insufficient to specify a goal of therapy. Furthermore, currently available drugs do not robustly raise HDL cholesterol. Nonetheless, a low HDL should receive clinical attention and management according to the following sequence. In all persons with low HDL cholesterol, the primary target of therapy is LDL

cholesterol; ATP III guidelines should be followed to achieve the LDL cholesterol goal. Second, after the LDL goal has been reached, emphasis shifts to weight reduction and increased physical activity (when the metabolic syndrome is present). When a low HDL cholesterol is associated with high triglycerides (200-499 mg/dL), secondary priority goes to achieving the non-HDL cholesterol goal, as outlined before. Also, if triglycerides are <200 mg/dL (isolated low HDL cholesterol), drugs for HDL raising (fibrates or nicotinic acid) can be considered; however, treatment for isolated low HDL is mostly reserved for persons with CHD and CHD risk equivalents.

Diabetic dyslipidemia. This disorder is essentially atherogenic dyslipidemia (high triglycerides, low HDL, and small dense LDL) in persons with type 2 diabetes. Although elevated triglycerides and/or low HDL cholesterol are common in persons with diabetes, clinical trial results support the identification of LDL cholesterol as the primary target of therapy, as it is in those without diabetes. Since diabetes is designated a CHD risk equivalent in ATP III, the LDL cholesterol goal of therapy for most persons with diabetes will be <100 mg/dL. Furthermore, when LDL cholesterol is ≥130 mg/dL, most persons with diabetes will require initiation of LDL-lowering drugs simultaneously with TLC to achieve the LDL goal. When LDL cholesterol levels are in the range of 100-129 mg/dL at baseline or on treatment, several therapeutic options are available: increasing intensity of LDL-lowering therapy, adding a drug to modify atherogenic dyslipidemia (fibrate or nicotinic acid), or intensifying control of other risk factors including hyperglycemia. When triglyceride levels are ≥200 mg/dL, non-HDL cholesterol becomes a secondary target of cholesterol-lowering therapy. Several ongoing clinical trials (e.g., Antihypertensive and Lipid Lowering Heart Attack Trial [ALLHAT]) will better quantify the magnitude of the benefit of LDL-lowering treatment in older individuals with diabetes. In older persons (≥65 years of age) with diabetes but no additional CHD risk factors other than age, clinical judgment is required for how intensively to apply these guidelines; a variety of factors, including concomitant illnesses, general health status, and social issues may influence treatment decisions and may suggest a more conservative approach.

Special Considerations for Different Population Groups

Middle-aged men (35-65 years). In general, men have a higher risk for CHD than do women. Middle-aged men in particular have a high prevalence of the major risk factors and are predisposed to abdominal obesity and the metabolic syndrome. A sizable fraction of all CHD in men occurs in middle age. Thus, many middle-aged men carry a relatively high risk for CHD, and for those who do, intensive LDL-lowering therapy is needed.

Women (ages 45-75 years). In women, onset of CHD generally is delayed by some 10-15 years compared with that in men; thus most CHD in women occurs after age 65. All risk factors contribute to CHD in women, and most premature CHD in women (<65 years) occurs in those with multiple risk factors and the metabolic syndrome. Despite the previous belief that the gender difference in risk for CHD reflects a protective effect of estrogen in women, recent secondary and primary prevention trials cast doubt on the use of hormone replacement therapy to reduce CHD risk in postmenopausal women. In contrast, the favorable effects of statin therapy in women in clinical trials make a cholesterol-lowering drug preferable to hormone replacement therapy for CHD risk reduction. Women should be treated similarly to men for secondary prevention. For primary prevention, ATP III's general approach is similarly applicable for women and men. However, the later onset of CHD for women in general should be factored into clinical decisions about use of cholesterol-lowering drugs.

Older adults (men ≥ 65 years and women ≥ 75 years). Overall, most new CHD events and most coronary deaths occur in older persons (≥ 65 years). A high level of LDL cholesterol and low HDL cholesterol still carry predictive power for the development of CHD in older persons. Nevertheless, the finding of advanced subclinical atherosclerosis by noninvasive testing can be helpful for confirming the presence of high risk in older persons. Secondary prevention trials with statins have included a sizable number of older persons, mostly in the age range of 65 to 75 years. In these trials, older persons showed significant risk reduction with statin therapy. Thus, no hard-and-fast age restrictions appear necessary when selecting persons with established CHD for LDL-lowering therapy. For primary prevention, TLC is the first line of therapy for older persons. However, LDL-lowering drugs can also be considered when older persons are at higher risk because of multiple risk factors or advanced subclinical atherosclerosis.

Younger adults (men 20-35 years; women 20-45 years). CHD is rare except in those with severe risk factors, e.g., familial hypercholesterolemia, heavy cigarette smoking, or diabetes. Even though clinical CHD is relatively rare in young adults, coronary atherosclerosis in its early stages may progress rapidly. The rate of development of coronary atherosclerosis earlier in life correlates with the major risk factors. In particular, long-term prospective studies reveal that elevated serum cholesterol detected in young adulthood predicts a higher rate of premature CHD in middle age. Thus, risk factor identification in young adults is an important aim for long-term prevention. The combination of early detection and early intervention on elevated LDL cholesterol with life-habit changes offers the opportunity for delaying or preventing onset of CHD later in life. For young adults with LDL cholesterol levels ≥130 mg/dL, TLC should be instituted and emphasized.

Particular attention should be given to young men who smoke and have a high LDL cholesterol (160-189 mg/dL); they may be candidates for LDL-lowering drugs. When young adults have very high LDL cholesterol levels (≥190 mg/dL), drug therapy should be considered, as in other adults. Those with severe genetic forms of hypercholesterolemia may require LDL-lowering drugs in combination (e.g., statin + bile acid sequestrant).

Racial and ethnic groups. African Americans have the highest overall CHD mortality rate and the highest out-of-hospital coronary death rates of any ethnic group in the United States, particularly at younger ages. Although the reasons for the excess CHD mortality among African Americans have not been fully elucidated, it can be accounted for, at least in part, by the high prevalence of coronary risk factors. Hypertension, left ventricular hypertrophy, diabetes mellitus, cigarette smoking, obesity, physical inactivity, and multiple CHD risk factors all occur more frequently in African Americans than in whites. Other ethnic groups and minority populations in the United States include Hispanics, Native Americans, Asian and Pacific Islanders, and South Asians. Although limited data suggest that racial and ethnic groups vary somewhat in baseline risk for CHD, this evidence did not appear sufficient to lead the ATP III panel to modify general recommendations for cholesterol management in these populations.

Adherence to LDL-Lowering Therapy

Adherence to the ATP III guidelines by both patients and providers is a key to approximating the magnitude of the benefits demonstrated in clinical trials of cholesterol lowering. Adherence issues have to be addressed in order to attain the highest possible levels of CHD risk reduction. Thus, ATP III recommends the use of state-of-the-art multidisciplinary methods targeting the patient, providers, and health delivery systems to achieve the full population effectiveness of the guidelines for primary and secondary prevention (Table 10).

Table 10. Interventions to Improve Adherence

Focus on the Patient

- Simplify medication regimens
- Provide explicit patient instruction and use good counseling techniques to teach the patient how to follow the prescribed treatment
- Encourage the use of prompts to help patients remember treatment regimens
- Use systems to reinforce adherence and maintain contact with the patient
- Encourage the support of family and friends
- Reinforce and reward adherence
- Increase visits for patients unable to achieve treatment goal
- Increase the convenience and access to care
- Involve patients in their care through self-monitoring

Focus on the Physician and Medical Office

- Teach physicians to implement lipid treatment guidelines
- Use reminders to prompt physicians to attend to lipid management
- Identify a patient advocate in the office to help deliver or prompt care
- Use patients to prompt preventive care
- Develop a standardized treatment plan to structure care
- Use feedback from past performance to foster change in future care
- Remind patients of appointments and follow-up missed appointments

Focus on the Health Delivery System

- Provide lipid management through a lipid clinic
- Utilize case management by nurses
- Deploy telemedicine
- Utilize the collaborative care of pharmacists
- Execute critical care pathways in hospitals

Appendix

Shared Features of ATP III and ATP II

ATP III shares a set of core features with ATP II. These are shown in Table A.

Table A. Shared Features of ATP III and ATP II

- Continued identification of LDL cholesterol lowering as the primary goal of therapy
- Consideration of high LDL cholesterol (≥160 mg/dL) as a potential target for LDL-lowering drug therapy, specifically as follows:
  - For persons with multiple risk factors whose LDL levels are high (≥160 mg/dL) after dietary therapy, consideration of drug therapy is recommended
  - For persons with 0-1 risk factor, consideration of drug therapy (after dietary therapy) is optional for LDL 160-189 mg/dL and recommended for LDL ≥190 mg/dL
- Emphasis on intensive LDL-lowering therapy in persons with established CHD
- Identification of three categories of risk for different LDL goals and different intensities of LDL-lowering therapy:
  - CHD and CHD risk equivalents* (other forms of clinical atherosclerotic disease)
  - Multiple (2+) risk factors†
  - 0-1 risk factor
- Identification of subpopulations, besides middle-aged men, for detection of high LDL cholesterol (and other lipid risk factors) and for clinical intervention. These include:
  - Young adults
  - Postmenopausal women
  - Older persons
- Emphasis on weight loss and physical activity to enhance risk reduction in persons with elevated LDL cholesterol

* *A CHD risk equivalent is a condition that carries an absolute risk for developing new CHD equal to the risk for having recurrent CHD events in persons with established CHD.*

† *Risk factors that continue to modify the LDL goal include cigarette smoking, hypertension, low HDL cholesterol, family history of premature CHD, age (male ≥45 years and female ≥55 years), and diabetes (in ATP III diabetes is regarded as a CHD risk equivalent).*

Estimating 10-Year Risk for Men and Women

Risk assessment for determining the 10-year risk for developing CHD is carried out using Framingham risk scoring (Table B1 for men and Table B2 for women). The risk factors included in the Framingham calculation of 10-year risk are: age, total cholesterol, HDL cholesterol, systolic blood pressure, treatment for hypertension, and cigarette smoking. The first step is to calculate the number of points for each risk factor. For initial assessment, values for total cholesterol and HDL cholesterol are required. Because of a larger database, Framingham estimates are more robust for total cholesterol than for LDL cholesterol. Note, however, that the LDL cholesterol level remains the primary target of therapy. Total cholesterol and HDL cholesterol values should be the average of at least two measurements obtained from lipoprotein analysis. The blood pressure value used is that obtained at the time of assessment, regardless of whether the person is on anti-hypertensive therapy. However, if the person is on antihypertensive treatment, an extra point is added beyond points for the blood pressure reading because treated hypertension carries residual risk (see Tables B1 and B2). The average of several blood pressure measurements, as recommended by the Joint National Committee (JNC), is needed for an accurate measure of baseline blood pressure. The designation "smoker" means any cigarette smoking in the past month. The total risk score sums the points for each risk factor. The 10-year risk for myocardial infarction and coronary death (hard CHD) is estimated from total points, and the person is categorized according to absolute 10-year risk as indicated above (see Table 5).

Table B1. Estimate of 10-Year Risk for Men (Framingham Point Scores)

Age	Points
20-34	-9
35-39	-4
40-44	0
45-49	3
50-54	6
55-59	8
60-64	10
65-69	11
70-74	12
75-79	13

Total Cholesterol	Points				
	Age 20-39	Age 40-49	Age 50-59	Age 60-69	Age 70-79
<160	0	0	0	0	0
160-199	4	3	2	1	0
200-239	7	5	3	1	0
240-279	9	6	4	2	1
≥280	11	8	5	3	1

	Points				
	Age 20-39	Age 40-49	Age 50-59	Age 60-69	Age 70-79
Nonsmoker	0	0	0	0	0
Smoker	8	5	3	1	1

HDL (mg/dL)	Points
≥60	-1
50-59	0
40-49	1
<40	2

Systolic BP (mmHg)	If Untreated	If Treated
<120	0	0
120-129	0	1
130-139	1	2
140-159	1	2
≥160	2	3

Point Total	10-Year Risk %
<0	< 1
0	1
1	1
2	1
3	1
4	1
5	2
6	2
7	3
8	4
9	5
10	6
11	8
12	10
13	12
14	16
15	20
16	25
≥17	≥ 30

Table B2. Estimate of 10-Year Risk for Women (Framingham Point Scores)

Age	Points
20-34	-7
35-39	-3
40-44	0
45-49	3
50-54	6
55-59	8
60-64	10
65-69	12
70-74	14
75-79	16

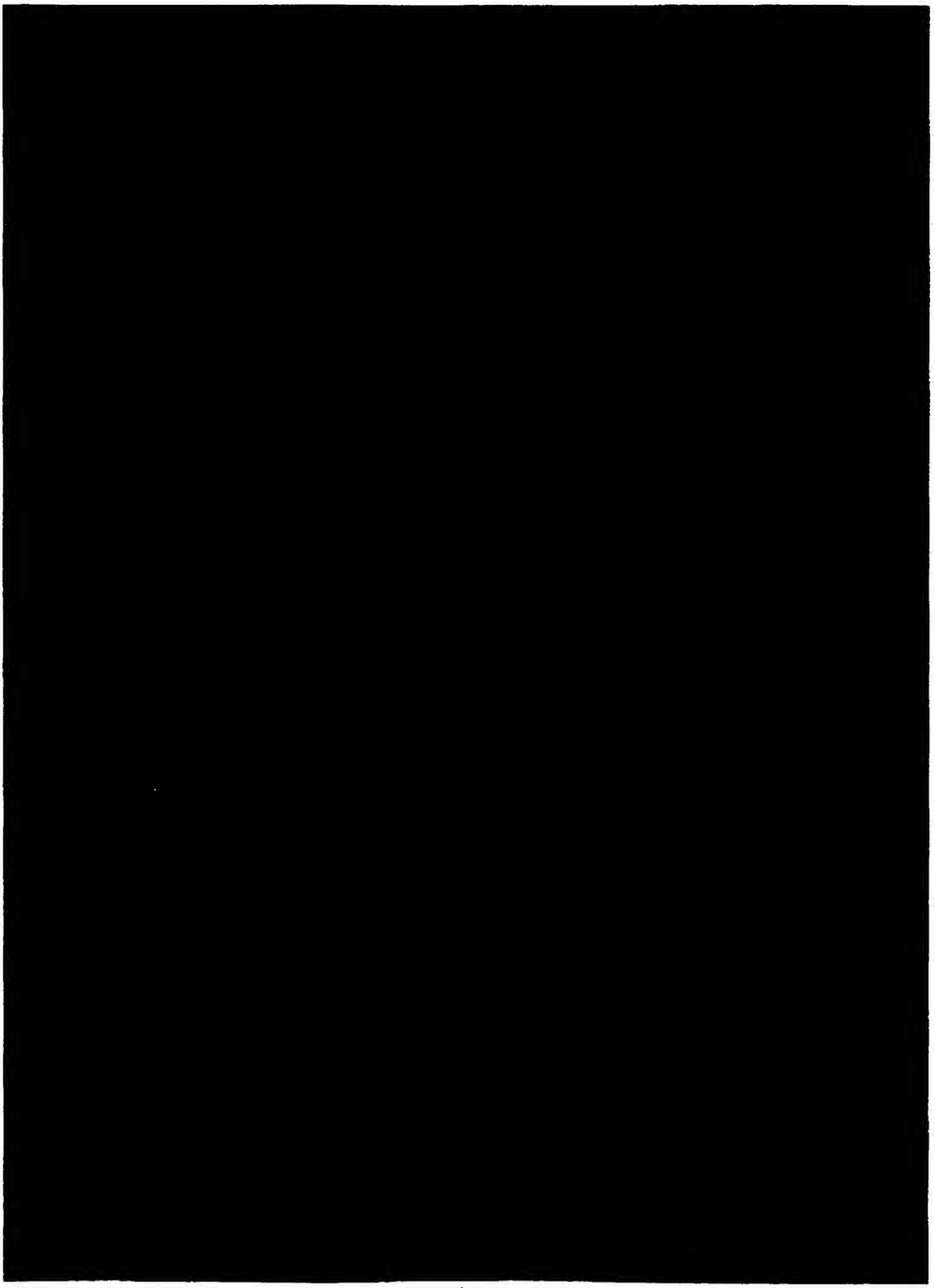
Total Cholesterol	Points				
	Age 20-39	Age 40-49	Age 50-59	Age 60-69	Age 70-79
<160	0	0	0	0	0
160-199	4	3	2	1	1
200-239	8	6	4	2	1
240-279	11	8	5	3	2
≥280	13	10	7	4	2

	Points				
	Age 20-39	Age 40-49	Age 50-59	Age 60-69	Age 70-79
Nonsmoker	0	0	0	0	0
Smoker	9	7	4	2	1

HDL (mg/dL)	Points
≥60	-1
50-59	0
40-49	1
<40	2

Systolic BP (mmHg)	If Untreated	If Treated
<120	0	0
120-129	1	3
130-139	2	4
140-159	3	5
≥160	4	6

Point Total	10-Year Risk %
<9	< 1
9	1
10	1
11	1
12	1
13	2
14	2
15	3
16	4
17	5
18	6
19	8
20	11
21	14
22	17
23	22
24	27
≥25	≥ 30



U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
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National Heart, Lung, and Blood Institute

NIH Publication No. 01-3670
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EXHIBIT I

Applicants: Jacob Bar-Tana
Serial No.: 10/735,439
Filed: December 11, 2003

Exhibit I

Brief Reports

Nicotinic Acid as Therapy for Dyslipidemia in Non–Insulin-Dependent Diabetes Mellitus

Abhimanyu Garg, MBBS, MD; Scott M Grundy MD, PhD

Recently, nicotinic acid has been recommended as a first-line hypolipidemic drug. To determine the effectiveness of nicotinic acid in dyslipidemic patients with non–insulin-dependent diabetes mellitus, 13 patients were treated in a randomized crossover trial. Patients received either nicotinic acid (1.5 g three times daily) or no therapy (control period) for 8 weeks each. Compared with the control period, nicotinic acid therapy reduced the plasma total cholesterol level by 24%, plasma triglyceride level by 45%, very-low-density lipoprotein cholesterol level by 58%, and low-density lipoprotein cholesterol level by 15%, and it increased the high-density lipoprotein cholesterol level by 34%. However, nicotinic acid therapy resulted in the deterioration of glycemic control, as evidenced by a 18% increase in mean plasma glucose concentrations, a 21% increase in glycosylated hemoglobin levels, and the induction of marked glycosuria in some patients. Furthermore, a consistent increase in plasma uric acid levels was observed. Therefore, despite improvement in lipid and lipoprotein concentrations, because of worsening hyperglycemia and the development of hyperuricemia, nicotinic acid must be used with caution in patients with non–insulin-dependent diabetes mellitus with dyslipidemia. We suggest that the drug not be used as a first-line hypolipidemic drug in patients with non–insulin-dependent diabetes mellitus.

(*JAMA* 1990;264:723-726)

DYSLIPIDEMIA is a common finding in non–insulin-dependent diabetes mellitus (NIDDM)[1] and probably contributes causally to coronary heart disease, a major cause of death in patients with NIDDM.[2] Recently, the National Cholesterol Education Program proposed new guidelines for the management of high blood cholesterol levels.[3] The guidelines obviously could not consider in depth every subgroup of patients with hyperlipidemias, and, therefore, problems of management of lipid and lipoprotein abnormalities in patients with NIDDM were not addressed in detail. The National Cholesterol Education Program recommended nicotinic acid and bile acid binding resins as first-line drugs for treatment of hypercholesterolemia, and nicotinic acid was designated as the drug of choice for hypercholesterolemic patients with concurrent hypertriglyceridemia. Since hypertriglyceridemia is the most prevalent lipid abnormality in NIDDM, the guidelines could be interpreted to mean that nicotinic acid is the drug of choice for diabetic dyslipidemia. Patients with NIDDM, however, have other metabolic abnormalities, and, therefore, the choice of hypolipidemic drug may not be the same as in nondiabetic patients. The purpose of this study was to examine the potential usefulness of nicotinic acid for the treatment of dyslipidemia in patients with NIDDM.

From the Veterans Administration Medical Center, Dallas (Drs Garg and Grundy), and the Center for Human Nutrition (Drs Garg and Grundy) and the Departments of Clinical Nutrition (Drs Garg and Grundy), Internal Medicine (Drs Garg and Grundy), and Biochemistry (Dr Grundy), University of Texas Southwestern Medical Center at Dallas.

Reprint requests to Center for Human Nutrition, 5323 Harry Hines Blvd, Dallas, TX 75235-9052 (Dr Grundy).

PATIENTS AND METHODS

Patients

Thirteen male patients with NIDDM from a lipid clinic and a diabetes clinic were studied at the Veterans Administration Medical Center, Dallas, Tex. All patients had an insidious onset of diabetes after age 38 years, and none had a history of ketosis. Their ages ranged from 49 to 68 years (mean ± SEM, 59 ± 1 years). Body weights and body-mass indexes averaged 91.7 ± 3.8 kg and 29.9 ± 0.7 kg/m², respectively. Four patients were receiving glyburide therapy, eight patients were receiving a combination of isophane insulin suspension and regular human insulin (Squibb-Novo, Princeton, NJ) subcutaneously before breakfast and supper for glycemic control, and one patient was receiving dietary therapy only. C-peptide levels were determined for patients receiving insulin therapy, both in the fasting state and 90 minutes after they ingested 480 mL of Sustacal (Mead Johnson & Co, Evansville, Ind); average values were 751 ± 182 and 1388 ± 192 pmol/L, respectively, confirming the diagnosis of NIDDM.[4] At entry, all patients had a plasma cholesterol level of 5.2 mmol/L or greater and/or a plasma triglyceride level of 2.8 mmol/L or greater. Six patients had coronary heart disease but none had recent myocardial infarction, unstable angina pectoris, or congestive heart failure. Patients were excluded if they had a history of peptic ulcer or gout, evidence of hyperuricemia (plasma uric acid concentration >475 μmol/L), or abnormal test results for liver, kidney, or thyroid gland functions. For patients taking specific hypolipidemic drugs, such therapy was discontinued at least 2 months prior to the study.

Experimental Design

The study protocol was approved by the institutional review board, and each patient gave informed consent. All patients were studied during three hospitalizations in the metabolic ward, each lasting 5 days. Before being randomized, patients were hospitalized for 5 days, called the baseline period, during which the dosage of insulin or glyburide was adjusted to achieve good glycemic control, and energy intake was determined to project a constant body weight. Thereafter, no changes in the dosage of insulin or glyburide were allowed except to prevent symptomatic hypoglycemia. The plasma glucose con-

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U.S. Serial No.: 10/735,439
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Exhibit I

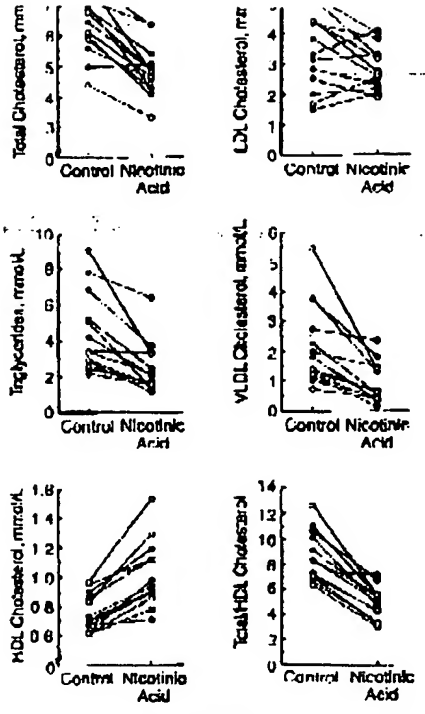

Fig 1.—Plasma levels of total cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, very-low-density lipoprotein (VLDL) cholesterol, and high-density lipoprotein (HDL) cholesterol and the ratio of total to HDL cholesterol during the control and the nicotinic acid periods in 18 patients with non–insulin-dependent diabetes mellitus with dyslipidemia. Each circle represents the mean of five daily determinations. Solid circles indicate mean values in patients receiving insulin therapy; open circles, values in patients receiving glyburide therapy or diet alone

centration was measured at 3, 7, and 11 AM and 4 and 9 PM each day. Fasting blood samples were drawn daily for analysis of lipids and lipoproteins. Blood was also drawn for a glycosylated hemoglobin determination and a routine hematologic and chemistry profile, including the uric acid concentration. Patients were instructed to follow an isocaloric diet throughout the study, the diet containing 50% carbohydrates, 30% fat, and 20% protein, with 300 mg of cholesterol.[8] They were instructed not to consume alcohol during the trial.

After the baseline hospitalization, patients were randomized to receive nicotinic acid or no therapy for a period of 8 weeks. All patients then crossed over to the drug/no therapy (control) period for the next 8 weeks. A double-blind, placebo-controlled trial was not planned because of previous reports of the ineffective nature of this design due to symptomatic side effects of nicotinic acid therapy.[8] The nicotinic acid dosage was gradually increased from 50 mg three times daily on the first day to 1.5 g three times per day by the end of third week. Thereafter, patients continued to take the full dosage for the next 5 weeks. Patients reported as outpatients at two weekly intervals for a chemistry profile. On day 51 of each period, the patients entered the metabolic ward for 5 days, and blood samples were obtained each day as described above. On the last day of each period, plasma specimens were obtained every 2 hours for the determination of glucose levels. The patients were also interviewed about the side effects of the medication, such as flushing, rash, gastrointestinal distress, allergic reactions, and gout.

	Baseline	Control	Nicotinic Acid	P†
Plasma cholesterol, mmol/L	6.71 ± 0.33	6.35 ± 0.28	4.82 ± 0.29	.0001
Plasma triglycerides, mmol/L	5.08 ± 0.84	4.46 ± 0.62	2.45 ± 0.40	.0008
VLDL cholesterol, mmol/L	2.57 ± 0.40	2.19 ± 0.38	0.91 ± 0.19	.0009
LDL cholesterol, mmol/L	3.39 ± 0.42	3.40 ± 0.38	2.89 ± 0.21	.07
HDL cholesterol, mmol/L	0.76 ± 0.05	0.76 ± 0.04	1.02 ± 0.06	.0001
Total cholesterol/HDL cholesterol	9.22 ± 0.61	8.58 ± 0.56	4.93 ± 0.35	.0001

*VLDL indicates very-low-density lipoprotein, LDL, low-density lipoprotein; and HDL, high-density lipoprotein. Values are mean ± SEM. To convert values from millimoles per liter to milligrams per deciliter, multiply the cholesterol values by 38.67 and the triglyceride values by 88.574.
†Comparison between the control and nicotinic acid periods by a two-tailed paired *t* test.

Biochemical Analyses

Fasting plasma samples were analyzed for total cholesterol, triglyceride, and lipoprotein cholesterol concentrations according to Lipid Research Clinic procedures,[7] except that cholesterol and triglyceride concentrations were measured enzymatically.[9,10] Briefly, very-low-density lipoprotein (VLDL, density <1.006 kg/L) was removed by preparative ultracentrifugation, and the cholesterol level was measured in the VLDL subfraction and the infranatant. The high-density lipoprotein (HDL) cholesterol level was measured in the supernatant after lipoproteins containing apolipoprotein B were precipitated by heparin-manganese.[7] Cholesterol in the low-density lipoprotein (LDL) fraction was taken as the difference between the cholesterol content of the 1.006-kg/L infranatant and HDL cholesterol.

The plasma glucose concentration was determined by glucose oxidase method using a glucose analyzer (Beckman Instruments Inc, Fullerton, Calif). Quantitative analysis of glycosylated hemoglobin was done by agar gel electrophoresis using kits (Helena Laboratories, Beaumont, Tex). The plasma C-peptide concentration was measured by radioimmunoassay kits (Mallinckrodt Inc, St Louis, Mo).

Statistical Analyses

A repeated-measures analysis of variance test was performed to compare the baseline, nicotinic acid, and control periods, to assess the effect of the sequence in which the patients were assigned to the control or active drug period, and to assess differences in response between patients receiving insulin and other therapy.[11,12] Multiple comparisons were made with use of the two-tailed paired *t* test with Bonferroni's correction. When three periods were included in the analysis, $P<.0167$ was considered significant. The Wilcoxon signed rank test was used for data not consistent with the hypothesis of normality. The areas under the curve were compared with use of a *t* test. All results are expressed as mean ± SEM.

RESULTS

The analysis of variance did not reveal any differences in the response to nicotinic acid therapy whether patients received insulin, glyburide, or no hypoglycemic drugs; therefore, plasma lipid and lipoprotein values in all patients were pooled. Results for each patient are shown in Fig 1, and results for all patients are summarized in Table 1. The order in which patients were allocated to the drug and control periods had no effect on the results. Plasma lipid and lipoprotein concentrations were not significantly different in the baseline and control periods (Table 1).

Compared with the control period, nicotinic acid therapy reduced the plasma total cholesterol level by 24%. Plasma triglyceride levels were reduced by 45% ($P<.001$) and VLDL cholesterol levels by 58% ($P<.001$). The LDL cholesterol levels showed a modest 15% decrease with nicotinic acid therapy which approached statistical significance ($P=.07$). The HDL cholesterol concentrations rose consistently, with

Table 2.—Metabolic Variables During the Study*

	Study Period			
	Baseline	Control	Nicotinic Acid	P†
Mean plasma glucose, mmol/L‡	7.19±0.31	7.85±0.50	9.09±0.63	.068
24-h plasma glucose profile mmol•h/L§		184.1±6.2	213.5±7.4	.047
Insulin dosage (n=9), U/d	85.4±9.5	87.4±10.0	91.6±10.2	.50
Glyburide dosage (n=4), mg/d	6.25±3.0	2.50±1.0	6.25±3.0	1.0
Glycosylated hemoglobin, %	9.6±0.4	9.7±0.6	10.5±0.5	.002
24-h urinary glucose, g/d	3.1±1.3	3.6±1.4	14.8±6.3	.016
Plasma uric acid, μmol/L‖	398±22	382±22	511±33	.0001
Body weight, kg	91.7±3.3	92.1±2.9	91.1±2.8	.19

*Values are mean ± SEM.
†Comparison between the control and nicotinic acid periods by a two-tailed paired *t* test.
‡Plasma glucose values were determined at 3 7, and 11 AM and 4 and 9 PM for 5 days during hospitalization. To convert values from millimoles per liter to milligrams per deciliter multiply by 18.02.
§Plasma glucose values were determined at 2-hour intervals for 24 hours on the last day of hospitalization in nine patients. Values are given in area-under-the-curve units.
‖To convert plasma uric acid values from micromoles per liter to milligrams per deciliter multiply by 0.0168.

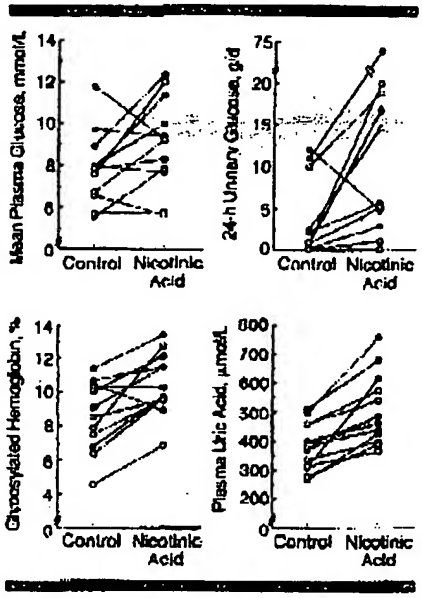

Fig 2.—Mean plasma glucose, 24-hour urinary glucose, glycosylated hemoglobin, and plasma uric acid levels during the control and the nicotinic acid periods in 13 patients with non–insulin-dependent diabetes mellitus with dyslipidemia. Solid circles indicate mean values in patients receiving insulin therapy, open circles, values in patients receiving glyburide therapy or diet alone.

an average increase of 34% ($P<.0001$). The ratio of total cholesterol to HDL cholesterol also improved strikingly during nicotinic acid therapy.

The daily requirements of hypoglycemic drugs did not change in 10 of 13 patients. In one patient, due to mild hypoglycemic episodes in the control period, the daily insulin dosage was reduced by 4 U. In another patient, glyburide therapy was discontinued due to persistently low blood glucose levels reported on self-monitoring during the control period. In the third patient, nicotinic acid therapy caused an unanticipated marked deterioration in fasting plasma glucose values (from an average of 8.2 mmol/L during the control period to 18.2 and 21.5 mmol/L during the outpatient follow-up with nicotinic acid therapy), necessitating an increase in the daily insulin dosage from 76 to 105 U. Despite a 38% increase in the daily insulin dosage, the patient's mean plasma glucose values during hospitalization remained elevated (11.3 mmol/L) during nicotinic acid therapy compared with values during the control period (7.8 mmol/L). Although the increase in insulin dosage in this patient did not follow the original protocol, he was not excluded from analysis.

Overall, glycemic control deteriorated during nicotinic acid therapy, as evidenced by a 16% increase in mean plasma glucose levels, from 7.8 to 9.1 mmol/L. Concentrations of glycosylated hemoglobin increased by 21% during nicotinic acid therapy, and marked glycosuria was noted in some patients (Table 2 and Fig 2). A daylong profile of plasma glucose, obtained on the last day of each period in nine patients, also revealed significantly higher values during nicotinic acid therapy (Table 2).

Nicotinic acid therapy increased plasma uric acid levels in all the patients (Table 2 and Fig 2). No patient, however, suffered from acute gouty arthritis. In two patients, mean plasma uric acid values rose to extremely high levels—684 and 761 μmol/L—with nicotinic acid therapy (Fig 2). Both of these patients had borderline low values of creatinine clearance, 1.05 and 1.08 mL/s, respectively, at entry into the study. A slight increase in the plasma creatinine concentration and a reduction in creatinine clearance was also noted in both patients during nicotinic acid therapy. No changes in the plasma creatinine concentration or creatinine clearance were noted in any other patients.

Most patients tolerated nicotinic acid therapy well except for minor complaints of flushing. None developed significant abnormalities in hepatic function test results throughout the study. One patient reported headaches but also noted improvement in claudication distance during nicotinic acid therapy. No patient dropped out as a consequence of side effects.

COMMENT

Soon after the discovery of the plasma lipid-lowering potential of nicotinic acid therapy by Altschul et al,[10] deterioration of glucose tolerance with this agent was reported in both nondiabetic subjects[12-14] and patients with NIDDM.[15,16] Since most of these claims were anecdotal, the potential clinical significance of this side effect has not been given due consideration. For instance, recent guidelines of the National Cholesterol Education Program can be taken to indicate that nicotinic acid is the drug of choice for treatment of dyslipidemia associated with NIDDM.[5] The current investigation, therefore, was carried out to examine carefully whether nicotinic acid will favorably modify plasma lipid and lipoprotein concentrations in patients with NIDDM without significantly worsening their glycemic control.

In our patients, nicotinic acid therapy was highly effective for lowering levels of plasma triglycerides and VLDL cholesterol. It also raised levels of HDL cholesterol, with an increase averaging 34%. Total cholesterol levels were reduced significantly, as were total/HDL cholesterol ratios. Nicotinic acid therapy reduced LDL cholesterol levels in most but not all patients. Still, it generally *did not* produce the rise in LDL cholesterol concentrations commonly observed with other triglyceride-lowering therapies, eg, fibric acids[14,19] or n-3 polyunsaturated fatty acids.[20,21]

This study leaves little doubt that nicotinic acid therapy improves the lipoprotein profile in patients with NIDDM. On the other hand, the drug also causes a deterioration in glycemic control. In almost all patients, levels of glycosylated hemoglobin rose with nicotinic acid therapy. The daily profile of plasma glucose during hospitalization revealed an overall 16% elevation in mean plasma glucose levels during the nicotinic acid period. Finally, treatment with nicotinic acid induced marked glycosuria in some patients. Two-hourly profiles of plasma glucose on the last day of hospitalization also revealed elevated plasma glucose values during nicotinic

protein values during administration of nicotinic acid to patients with NIDDM may be counterbalanced by worsening hyperglycemia.

The results of our study suggest that a number of patients whose hyperglycemia is well controlled by dietary therapy alone may need to take hypoglycemic agents during nicotinic acid treatment. In others, the dosage of insulin or oral hypoglycemic drugs may have to be increased to control nicotinic acid–induced hyperglycemia. There are theoretical objections to increasing the insulin dosage for correction of metabolic derangements caused by another agent. For example, marked hyperinsulinemia may have a direct role in atherogenesis.[25] Furthermore, modest increases in insulin dosage may not be able to correct nicotinic acid–induced hyperglycemia, as was observed in one of the patients. Thus, it cannot be assumed that the worsening of hyperglycemia with nicotinic acid can be easily corrected by increasing the dosage of insulin or oral hypoglycemic drugs. Since the hyperglycemic action of nicotinic acid may be dose-dependent, some may argue that the dosage of nicotinic acid can be reduced if glycemic control deteriorates. However, the improvement in the lipoprotein profile likewise may not be optimal.

The mechanism for the hyperglycemic action of nicotinic acid in patients with NIDDM is not clear. Recently, it has been reported that nicotinic acid therapy may induce insulin resistance in normal, healthy volunteers.[26] The same could be true for patients with NIDDM. Whether nicotinic acid has any adverse effects on beta-cell function is not known, but there is no evidence to support such an action.[4,27] Another possibility is that, by interfering with triglyceride synthesis in the liver, nicotinic acid may enhance utilization of fatty acids at the expense of glucose; if so, this could lead to enhanced hepatic glucose output, another potential cause of hyperglycemia.

Another adverse effect of nicotinic acid therapy in this study was a consistent increase in plasma uric acid levels. Long-term therapy with nicotinic acid is known to increase the occurrence of acute gouty arthritis and to require greater usage of antigout medication.[4] Since patients with impaired glucose tolerance and NIDDM may be predisposed to develop hyperuricemia and gout,[28,29] nicotinic acid therapy may further increase the risk for development of gout. Although not all investigators agree that asymptomatic hyperuricemia is a risk factor, marked hyperuricemia caused by nicotinic acid therapy further compromised their renal function.

In summarize, nicotinic acid therapy markedly improves the lipoprotein profile of patients with NIDDM. Although nicotinic acid generally was well tolerated in the current patients, it is known to have a variety of side effects that preclude its use in many patients. For patients with NIDDM in particular, two side effects emerge as especially worrisome. First, the drug causes deterioration of glucose control, which, for long-term therapy, must be considered a definite drawback. Also, nicotinic acid raises uric acid levels, which increases the risk for gout and could have a negative effect on renal function. For most patients with NIDDM who have dyslipidemia, therefore, nicotinic acid therapy must be used with caution, although it may be useful in primary forms of dyslipidemia. On the basis of our previous studies, we suggest that a hydroxymethylglutaryl coenzyme A reductase inhibitor[27] or, for marked hypertriglyceridemia, a fibric acid derivative[19] may be preferable as a lipid-lowering drug. Further studies, however, are needed to identify the optimal pharmacologic approach to lipid lowering in patients with NIDDM.[29]

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EXHIBIT J

Applicants: Jacob Bar-Tana
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Exhibit J

The diabetogenic potential of thiazide-type diuretic and beta-blocker combinations in patients with hypertension

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Background **Recently published trials addressing the pharmacological management of hypertension have reported an increase in new-onset diabetes mellitus when comparing certain older and newer treatment regimens. Thiazide-type diuretics (thiazides) and beta-blockers have been individually implicated, but these drugs are frequently combined, and the magnitude of risk associated with their combined use has not been quantified.**

Methods and results **Randomized control trials were retrieved that: (i) featured stepped treatment to manage hypertension; (ii) compared initial treatment using a thiazide or beta-blocker (older drug) with an angiotensin-converting enzyme inhibitor, angiotensin receptor blocker or calcium antagonist (newer drug); (iii) assessed cardiovascular outcomes; (iv) reported new-onset diabetes; and (v) provided at least 1-year follow-up. A meta-analysis of available trials indicated that patients exposed to treatment regimens combining thiazides and beta-blockers are at greater risk of developing diabetes than regimens avoiding this combination of drugs (risk ratio for alternative therapy 0.81, 95% confidence interval 0.77–0.86). Current data cannot inform reliably about the risks associated with individual older drugs because of similar overall exposures in patients starting on newer and older drugs.**

Interpretation and implications **The results suggest that the routine combined use of a thiazide with a beta-blocker should be questioned in the early management of hypertension, particularly in patients who are at increased risk of developing new-onset diabetes. In such patients, the increased risk of developing diabetes may exceed the benefit of blood pressure lowering.**
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Keywords: hypertension, new-onset diabetes, drug therapy, thiazide-type diuretics, beta-blockers, iatrogenic harm

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Introduction

Hypertension drug trials commonly aim to reduce patient blood pressure to a target level applying protocols that variously offer a sequence of drugs and dose steps. Overviews of published trials consistently find similar improvements in cardiovascular outcomes for the major antihypertensive drug classes, regardless of the initial drug or sequence of drugs. However, evidence that thiazide-type diuretics (thiazides) and subsequently beta-blockers might impair glucose metabolism and induce diabetes has accumulated in each decade since the 1960s [1–6]. Pharmacologically this is plausible because beta-blockers increase insulin resistance and thiazides reduce insulin secretion [7–9]. Conversely, it has been argued that drugs that effectively inhibit the renin–angiotensin system may actually decrease insulin resistance and new-onset diabetes [10].

The findings of large-scale randomized controlled trials have recently been analysed to assess the risk of inducing diabetes with individual older drugs (thiazides or beta-blockers) [11–13], although the findings and interpretation have been contested [14]. It is possible that the combination of older drugs could further enhance the risk of new-onset diabetes, although this risk has not been formally evaluated or quantified. The issue is important because of the common combined use of thiazides and beta-blockers in the management of hypertension. A recent prospective study found that when diabetes developed during treatment for hypertension, the new-onset diabetes conveyed a risk of cardiovascular disease similar to that faced by the broader comparable population of patients with diabetes [15]. We sought to inform the question 'do beta-blockers and thiazide-type diuretics used in combination lead to an increased incidence of diabetes mellitus?' using published sources [16].

This paper clarifies the interpretation that can be placed on currently published trial data: the increased risk of combined (rather than individual) use of older drugs. It

Table 1 Brief detail of study selection and data abstraction

1. From medical database and published review searches, two reviewers independently selected studies and abstracted data.
2. Randomized controlled trials evaluating stepped drug treatment for essential hypertension were included if:
   (i) including an arm beginning with a thiazide-type diuretic or beta-blocker and subsequently adding the other drug when necessary;
   (ii) assessing cardiovascular outcomes;
   (iii) reporting new-onset diabetes; and
   (iv) of at least 1 year duration.
3. Patients in trials with diabetes at enrolment were excluded from our analysis.

enumerates the balance between potential benefit and harm in typical lower-risk patients now recommended for treatment in national guidelines; it describes the scope for publication bias by assessing published trials that do not contribute to the analysis, and makes clear research recommendations about how to resolve remaining controversies in this field. Preliminary findings from the Anglo-Scandinavian Cardiac Outcomes Trial Blood Pressure Lowering Arm (ASCOT-BPLA) [17] are discussed in the context of our findings.

Methods

By searching MEDLINE, EMBASE, Central, hypertension guidelines, systematic reviews and meta-analyses, we retrieved and abstracted data from hypertension treatment trials that met all set criteria (see Table 1). The criteria were selected so that the same trial that provided evidence of long-term benefit from treatment also informed the long-term risk of developing diabetes. Data on the quality of included trials, the characteristics of their participants and interventions, and relevant outcomes were independently abstracted by two reviewers. Any differences were resolved by discussion or by a third party. We estimated a pooled risk ratio for new-onset diabetes using a random effects model reflecting variation in the definition of new-onset diabetes applied in trials. The meta-analysis compared patients randomly assigned to either a thiazide or beta-blocker, combining their use if necessary, with patients receiving a different drug sequence beginning with a newer antihypertensive drug.

Results

Ten trials were identified that provided an appropriate treatment regimen to inform our study question [18–27]. Seven of these trials provided data on new-onset diabetes and could be included in our analysis. [18–24] (see Table 2). These seven trials provided 36 581 patients whose therapy began with an older drug and 40 368 who started on a newer drug (see Table 2). From the total pool of 10 trials, the analysis includes 76% of patients and 83% of patient years of treatment. The (crude) average rate of reported new-onset diabetes across trials during follow-up was 7.4 or 1.7% per year.

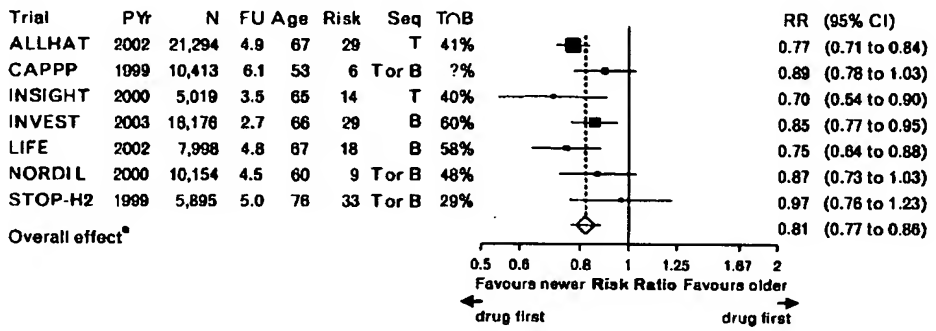
Overall, there was a significantly higher incidence of diabetes in patients randomly assigned to beta-blockers or thiazides of whom approximately half received both agents: the relative risk for newer versus older drug treatment = 0.81, 95% confidence interval (CI) 0.77–0.86 (see Fig. 1). The finding was robust when testing for heterogeneity ($Q = 9.04$; $P = 0.17$), variation of effect with study size ($P = 0.56$), and when using different methods of estimation. By visual inspection the findings provide no evidence to suggest that the relative risk relationship between combined use and new-onset diabetes changes with age, baseline risk, or the order in which the drugs are given. The number of studies is too few to explore confounding formally by meta-regression. Trials did not report new-onset diabetes data stratified for prognostic factors such as age, sex, ethnicity or body mass index, and it is not currently possible to explore whether

Table 2 Trials using thiazide-type diuretic and beta-blocker combination therapy and reporting incidence of new-onset diabetes mellitus

	Treatment regimen[a]		Older drugs combined		Incidence of diabetes[b]	
Trial	1st drug	Further drugs added	TD ∩ BB	I (TD ∪ BB)	During trial	Per year
ALLHAT	I1: CCB		0%	TD: 17%	9.8% (561/5726)	2.0%
	I2: ACE	BB or OD	0%	TD: 16%	8.1% (473/5842)	1.7%
	C: TD		41%	–	11.6% (1128/9727)	2.4%
CAPPP	I: ACE	TD then CCB	0%	BB: ?	6.5% (337/5183)	1.1%
	C: TD or BB	TD and BB then CCB	?	–	7.3% (380/5230)	1.2%
INSIGHT	I: CCB	BB (or ACE[c]) then OD	0%	BB: 38%	3.8% (96/2508)	1.1%
	C: TD		40%	–	5.5% (137/2511)	1.6%
INVEST	I: CCB	ACE then TD then OD	0%	TD: 41%	7.0% (569/8098)	2.6%
	C: BB	TD then ACE then OD	60%	–	8.2% (665/8078)	3.0%
LIFE	I: ARB	TD or OD	0%	TD: 62%	6.0% (241/4019)	1.3%
	C: BB		58%	–	8.0% (319/3979)	1.7%
NORDIL	I: CCB	ACE then TD or OD	0%	TD or BB: 30%	4.3% (216/5059)	0.9%
	C: TD or BB	TD and BB then ACE or OD	48%	–	4.9% (251/5095)	1.1%
STOP-H2	I1 ACE:	TD	0%	TD: 28%	4.7% (93/1969)	1.0%
	I2: CCB	BB	0%	TD: 30%	4.8% (95/1965)	1.0%
	C: TD or BB	TD and BB	29%	–	4.9% (97/1961)	1.0%

I, Trial arm starting with newer drug: ACE, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CCB, calcium antagonist; OD, other drug. C, Trial arm starting with older drug: BB, beta-blocker; TD, thiazide-type diuretic. TD ∩ BB, Proportion taking TD and BB in combination. TD ∪ BB, Proportion taking either a TD or BB as second or third-line therapy after starting on a newer drug. [a]Steps in dose are not shown. [b]Patients enrolled in trials with diabetes at baseline are excluded from the numbers shown. [c]If BB contraindicated.

Fig. 1



Meta-analysis of trials comparing antihypertensive therapy initiated with older and newer drugs: risk ratio for new-onset diabetes mellitus. Age, Average age of patients at enrolment; CI, confidence interval; FU, average trial follow-up in years; N, total number of patients enrolled without diabetes at baseline; PYr, publication year; Risk, baseline risk of all-cause mortality per 1000 patient-years for all patients enrolled; Seq, drug sequence in older drug arm in which T is a thiazide-type diuretic and B is a beta-blocker. T(T first, then B if necessary), B(B first, then T if necessary), T or B (either, then both if necessary); RR, relative risk; T ∩ B, approximate percentage in older drug arm receiving both T and B drugs. *Heterogeneity, $Q = 9.04$, $P = 0.17$. Normalized effect versus precision, $P = 0.56$.

the average finding varies with the known prognostic risk for developing diabetes.

Discussion

The results suggest that the routine combined use of a thiazide with a beta-blocker increases the risk of developing new-onset diabetes. We have conducted a secondary analysis of trials that included subgroups of patients randomly assigned, thus our analysis is potentially vulnerable to confounding and reporting bias. However, our meta-analysis is based on findings in nearly 77 000 patients, is biologically plausible, and is unlikely to be a consequence of publication bias.

Interpretation

An analysis of summary findings of these trials cannot explore the increased risk of using either a thiazide or beta-blocker as monotherapy. In trial arms of older drugs (see Table 2) approximately one-half of patients remain on single drug therapy (either a thiazide or beta-blocker), whereas in newer drug arms approximately one-third of patients subsequently use either a thiazide or beta-blocker. The single use of either drug is thus approximately balanced across newer and older treatment arms. As newer drugs are not associated with increased risk [28], the analysis for new-onset diabetes (approximately) compares combined thiazide–beta-blocker use with treatment using neither of these agents. If such an interpretation is accepted, then our findings have been diluted (by those in each arm receiving one older drug) and it can be hypothesized that a pure comparison of newer versus older drugs would find an even bigger diabetogenic effect.

The ASCOT-BPLA trial compares a 'newer' (calcium antagonist ± angiotensin-converting enzyme inhibitor) with an 'older' (beta-blocker ± thiazide) treatment regimen and will inform this issue. Preliminary reporting of the data indicates an excess of new diabetes in the older drug arm of this study of approximately 30%. This finding is consistent with the possibility that we may have underestimated the potential diabetogenic risk associated with a beta-blocker/thiazide combination [29]. In addition, one further small trial in treatment-naïve patients and not meeting our inclusion criteria, investigated the effect of newer and older drug-based therapies on metabolic parameters [30]. Its findings similarly suggested a greater increase in the risk of new-onset diabetes associated with the 'older' therapies: the risk ratio for newer versus older drug treatment was 0.125 (95% CI 0.003–0.70).

In our analysis, the incidence of diabetes varied across trials from 1 to 3% per year (i.e. 10–30 per 1000 patient-years of treatment). This may partly reflect baseline risk, but may also be caused by different definitions of new-onset diabetes. For example, ALLHAT, the largest trial, which featured a high absolute rate, applied a very inclusive definition: one reading of fasting serum glucose of 126 mg/dl or greater (≥ 7.0 mmol/l). However, we have used: (i) a random effects model in our meta-analysis to allow for the variability in estimation between trial populations; and (ii) the relative risk metric (comparing the relative risk of developing diabetes with newer and older therapies) as this is anticipated to be reasonably robust in the presence of differing definitions of new-onset diabetes.

The proportion of patients exposed to a thiazide–beta-blocker combination varied and was not always clearly reported: no estimation was possible for the CAPPP trial [18]. The time exposed to drug combinations was not reported for any trial and we have assumed that second-line therapies were initiated without substantial delay. We were unable to obtain further data from a number of published studies [18,20,22–24].

The issue of whether new-onset diabetes associated with taking the older drugs confers increased cardiovascular risk cannot be resolved from published trial data. While the numbers involved in trials are too small to see the adverse effect of extra cases of diabetes on cardiovascular trial endpoints, epidemiological investigation suggests these cases are at similar risk to the broader diabetic population [12,15].

Clinical importance

When national guidelines recommend treating raised blood pressure in patients at lower levels of cardiovascular risk, the absolute benefits of treatment are modest and thus the possible harm attributable to the drugs is particularly important. In the United States, the seventh report of the Joint National Committee recommends initiating therapy with a thiazide for most people, and beta-blockers will be a common second-line therapy [31]. Using a Framingham risk calculator, 60-year-old men with blood pressure 160/100 mmHg but without other cardiovascular risk factors and no evidence of target organ damage may typically face a 20% risk of cardiovascular disease over the next 10 years. Treatment for raised blood pressure in such patients is predicted to reduce cardiovascular events by approximately four per 1000 patient-years of treatment [32]. Assuming a 20% baseline risk of developing diabetes over the next 10 years (the mid-point of studies), our finding implies that a combination of a thiazide and beta-blocker may lead to an additional four cases of diabetes per 1000 patient-years of treatment. The balance between benefit and harm can thus sit uncomfortably close and may shift in favour of harm in those at highest risk of developing diabetes. The balance is also less favourable in women because of their lower average risk of cardiovascular disease. Sixty-year-old women (similarly with blood pressure 160/100 mmHg but without other cardiovascular risk factors) have a lower absolute risk of cardiovascular disease (14%) and thus lower average predicted benefit from treatment (three cardiovascular events prevented per 1000 patient-years of treatment).

New national guidelines such as those produced for and on behalf of the National Institute for Health and Clinical Excellence in the UK have begun to recognize these issues and have placed a limited caution against the combined use of beta-blockers with thiazides for those at highest risk of developing diabetes [33]. The guideline development group (constituted by representative healthcare professionals, patients and researchers) took the unanimous view that most primary care physicians would be unwilling to prescribe a thiazide and beta-blocker combination in the early stages of treatment for hypertension in patients at increased risk of developing new-onset diabetes. However, it was recognized that the combination might still be necessary as part of a treatment strategy for patients with treatment-resistant hypertension or if intolerance to other drug classes emerges. Patients at increased risk of developing diabetes were defined simply as those with a strong family history of type II diabetes, impaired glucose tolerance (fasting plasma glucose $\geq$ 6.5 mmol/l), those with clinical obesity (body mass index $\geq$ 30) or those of south-Asian or African-Caribbean ethnic origin. It would be interesting to compare the performance of this simple definition against more sophisticated criteria [34].

Future research

Our analysis, drawing upon summary published data, leaves important questions unanswered. For example, our estimation of the exposure times to drugs is necessarily approximate. A patient-level meta-analysis of published and future trials, providing prognostic data and exposure times to different agents, would allow the medical community to understand more about the diabetogenic potential of older drugs used alone or in combination, as well as in patients at a different underlying risk of developing diabetes.

In conclusion, the findings of this meta-analysis provide the first direct quantification of the potential of beta-blocker or thiazide-based treatment regimens, when used in combination, to enhance the risk of developing diabetes in individuals with hypertension. An assessment of the balance of risk and benefit suggests that the routine combined use of the older antihypertensive drugs should be avoided in the early management of hypertension in patients at increased risk of developing diabetes.

Contributors

J.M.M. designed the study, analysed the data and wrote the report. H.O.D. checked the study selection, data abstraction and the analysis. D.J.N. and F.C. performed study selection and data abstraction. B.W. and G.A.F. identified the need for this study, provided clinical guidance and helped interpret findings. All authors helped with the preparation of the report and approved the final submitted version. Ethical approval was not required for this study.

Conflict of interest statement

J.M.M. has previously received academic funding, fees and expenses for research and consultancy work from the UK Department of Health, medical charities and from

the pharmaceutical industry who manufacture treatments discussed in this report.

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G.A.F. and B.W. have received honoraria from a number of pharmaceutical companies for lectures and consultancy, and grant support for research studies and clinical trials from the pharmaceutical industry.

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